

**CONTRACTION-INDUCED GLUCOSE
TRANSPORT INTO SKELETAL MUSCLE: THE
INVOLVEMENT OF PROTEIN KINASE C**

by

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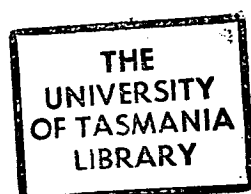


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ABSTRACT

The role of PKc as a possible intracellular mediator of exercise-induced increase in skeletal muscle glucose transport was investigated. Two skeletal systems were used, the *in vivo* rat gastrocnemius-plantaris-soleus muscle preparation, and the *in vitro* isolated soleus muscle preparation.

PKc was partially purified from gastrocnemius-plantaris-soleus skeletal muscle preparations by DE52 anion exchange chromatography. The skeletal muscle content of PKc was found to be higher than previously reported. Levels of PKc activity were found to be comparable to those of published PKa values, which suggest an equally important function for PKc in cellular events. The complete extraction of PKc from skeletal muscle membranes required a high concentration of Triton X100. This suggested that the hydrophobic domain of the enzyme was tightly bound to the membrane bilayer. Analysis of the intracellular distribution showed that a large proportion of the enzyme activity (60%) was associated with the membrane component of resting muscle.

Electrical-induced contraction of the gastrocnemius-plantaris-soleus muscle group resulted in a time-dependent translocation of PKc and a 2-fold increase in the concentration of both diacylglycerol and phosphatidic acid. The maximal values for the latter were reached at 2 min and preceded the maximum translocation of PKc (10 min). No PKm formation was detected by the production of a Ca^{2+} -, phospholipid-independent histone kinase activity. No reversal of

translocated PKc or decrease in the concentrations of diacylglycerol and phosphatidic acid occurred after 30 min of rest following a 5 min period of stimulation *in vivo*. The translocation of PKc was not influenced by variations in applied load at maximal fiber recruitment, but was dependent on the frequency of non-tetanic stimuli reaching a maximum at 4 Hz. Thus, it is concluded that electrical-induced muscle contraction increased the production of diacylglycerol and the translocation of PKc. This is indicative of enzyme activation.

The relationship between PKc and glucose transport was explored, *in vivo*, by varying the number of tetanic stimuli. Only one train of stimuli (200 ms, 100 Hz) was required for maximum translocation of PKc and for diacylglycerol and phosphatidic acid production. However, more than 35 trains of stimuli were required to activate glucose transport. The electrical stimulation of isolated soleus muscle *in vitro* increased the rate of glucose transport, but this required 20 min to reach maximum. Therefore, the time course for the activation of glucose transport is slower than PKc translocation. Thus, it is concluded that a "causal" relationship between electrical stimulation and the increase in glucose transport involving PKc activation may exist.

In an attempt to further clarify a role for PKc in the mechanism of glucose transport activation isolated soleus muscle was depleted ("downregulated") of PKc activity by prolonged TPA treatment. The response to both insulin and contraction in terms of glucose transport was assessed. Muscles treated with TPA showed an increased basal rate of 3-O-methylglucose uptake, responded partially to insulin,

but did not respond to contraction. The TPA treated and non-treated muscles were indistinguishable in terms of pre-contraction content of adenine nucleotide, creatine phosphate, lactate and glycogen, as well as contractile performance and contraction-induced glycogenolysis. Thus, it is concluded that treatment of isolated soleus muscle with TPA gives rise to a marked loss of contraction-induced glucose transport.

Overall, the work embodied by this thesis supports a case for the involvement of PKc in contraction-mediated uptake of glucose by skeletal muscle.

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ABBREVIATIONS

PKc	Protein Kinase C
PKm	Protein Kinase M
PKa	Protein Kinase A
TPA	12-0-tetradecanoylphorbol 13-acetate
DAG	Diacylglycerol
IP ₃	Inositol 1,4,5-trisphosphate
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
EGTA	[ethylenebis(aminoethyl ether)-N-tetraacetic acid
PI4,5-bisP	Phosphatidylinositol 4,5-bisphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid di-sodium salt
PDD	4a-phorbol 12,13-didecanoate
DMSO	Dimethylsulphoxide
[³ H]PDBu	[20(n)- ³ H]phorbol 12,13-dibutyrate
TLC	Thin layer chromatography
PCA	Perchloric Acid

PREFACE

Part of the work described in this thesis has been published. These publications are listed below in the order in which they were submitted and copies are attached in Appendix II.

1. RICHTER, E.A., CLELAND, P.J.F., RATTIGAN, S. AND CLARK, M.G. (1987) Contraction-associated translocation of protein kinase C in rat skeletal muscle. *FEBS Lett.* **217**: 232-236.

2. CLELAND, P.J.F., RICHTER, E.A., RATTIGAN, S., COLQUHOUN, E.Q. AND CLARK, M.G. (1989) Protein kinase C in rat skeletal muscle. In: *Cell Calcium Metabolism*. Plenum Press, N.Y., chapter 23, p. 211-219.

3. CLARK, M.G., CLELAND, P.J.F., AND RATTIGAN, S. (1989) Biochemistry of insulin and contractile effects in cardiac and skeletal muscle. In: *Pathophysiology of Insulin Action in Muscle*, Diabetes 1988 (Larkins, R., Zimimet, P, Chisholm, D., eds). Elsevier, N.Y.

4. CLELAND, P.J.F., APPLEBY, G.J., RATTIGAN, S. AND CLARK, M.G. (1989) Exercise-induced translocation of protein kinase C and production of diacylglycerol and phosphatidic acid in rat skeletal muscle *in vivo*: Relationship to changes in glucose transport. *J. Biol. Chem.* **264**: 17704-17711.

5. CLELAND, P.J.F., ABEL, K.C., RATTIGAN, S. AND CLARK, M.G. (1990) Long-term treatment of isolated rat soleus muscle with phorbol ester leads to loss of contraction-induced glucose transport. *Biochem. J.* **267**: 659-663.

The following papers have been presented at scientific meetings for which abstracts have been published.

1. CLELAND, P.J.F., RICHTER, E.A., RATTIGAN, S. AND CLARK, M.G. (1987) Contraction-associated translocation of protein kinase C in rat skeletal muscle. 7th International Washington Spring Symposium. In: *Cell Calcium Metabolism '87: Physiology, Biochemistry, Pharmacology and Clinical Applications*, p. 231, Washington D.C., U.S.A.

2. CLELAND, P.J.F., RATTIGAN, S., APPLEBY, G.J. AND CLARK, M.G. (1988) A role for protein kinase C in the insulin-like action of muscle contraction on glucose transport? *Proc. Biochem. Soc. Aust.* 20: S76.

3. CLELAND, P.J.F., RATTIGAN, S., APPLEBY, G.J. AND CLARK, M.G. (1988) Exercise-induced translocation of protein kinase C and production of diacylglycerol and phosphatidic acid in rat skeletal muscle *in vivo*. 7th International Biochemistry of Exercise Conference. *Can. J. Sport Sci.* 13: 9p.

4. CLELAND, P.J.F., ABEL, K.C., RATTIGAN, S. AND CLARK, M.G. (1989) Down-regulation of protein kinase C in isolated rat soleus muscle results in the loss of contraction-induced glucose transport. *Proc. Biochem. Soc. Aust.* 21: sp14.

This thesis contains no material which has been accepted for the award of any other higher degree or graduate diploma in any tertiary institution and that, to the best of my knowledge and belief, it contains no material previously published or written by another person, except when due reference is made in the text.

PERRY J.F. CLELAND

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Skeletal muscle represents a large portion of the body's mass (Laughlin and Armstrong, 1983) and accounts for the majority of glucose utilised during normal everyday activities (DeFronzo *et al.*, 1981). Therefore, skeletal muscle is considered to be of critical importance in maintaining blood glucose homeostasis.

Exercise-mediated glucose uptake into skeletal muscle has been recognized for some time (Chauveu and Kaufmann, 1887). However, the molecular mechanism still remains a mystery despite several theories (Wallberg-Henriksson, 1987).

1.2 GLUCOSE TRANSPORTER

The transport of glucose across the impermeable plasma membrane of the cell is mediated by specific mobile carrier proteins called transporters (Morgan *et al.*, 1964). These glucose transporters differ in various tissues. They are characterized by the different mechanisms by which they transport glucose across the plasma

membrane. There are two processes by which glucose is transported into cells by glucose transporters: (i) transporters that are found in the kidney and intestine require the co-transport of cations such as Na^+ or H^+ (Baly and Horuk, 1988); and (ii) transporters that operate by facilitated diffusion, represented by liver, red blood cells, fibroblast, adipocytes and muscle. The latter group of glucose transporters are relevant to this review. The glucose transporters that operate by facilitated diffusion can be further subdivided into insulin responsive (adipose and muscle) and insulin unresponsive groups.

A variety of glucose transporter proteins exist in the various tissues. These transporters vary in their K_m for glucose, isoelectric point and molecular weight which indicates that they have differing structural and functional properties.

The erythrocyte glucose transporter protein has a molecular weight of 55 kDa. The K_m for D-glucose was 1.6 mM as determined by equilibrium exchange (Baly and Horuk, 1988). The transport process is driven by the glucose concentration gradient and is therefore representative of a process of unregulated facilitated diffusion.

The liver glucose transporter has a molecular weight of between 45-50 kDa and a K_m for glucose of 17-30 mM (Baly and Horuk, 1988). This transporter serves to regulate plasma glucose levels and it functions via facilitated diffusion. Moreover, the liver glucose transporter is a high-capacity low-affinity carrier whose glucose transporter activity is in direct proportion to increases in blood glucose levels (Baly and Horuk, 1988).

The adipose glucose transporter has a molecular weight of between 42-47 kDa and a K_m for glucose of 5 mM (Baly and Horuk, 1988). Glucose uptake into these cells is regulated by insulin (Simpson and Cushman, 1986).

Glucose transport into skeletal muscle occurs by a process of regulated facilitated diffusion (Clausen, 1975; Elbrinck and Bihler, 1975). The two major regulators of glucose transport in skeletal muscle are insulin and muscle contraction. Extensive reviews on the effects of insulin on skeletal muscle transport can be found elsewhere (Kahn and Cushman, 1985; Simpson and Cushman, 1986; Wasserman and Vranic, 1986; Baly and Horuk, 1988). However, in this review, the present understanding on the effect of muscle contraction on glucose transport will be summarized.

1.3 SKELETAL MUSCLE GLUCOSE TRANSPORTER

The glucose transporter protein was identified in plasma membranes from rat skeletal muscle by using two methods. One was photolabelling with [^3H]cytochalasin B. This compound binds with high affinity to the cytoplasmic side of the glucose transporter protein. The second approach used rabbit antibodies against the purified human erythrocyte glucose transporter (Klip *et al.*, 1983). These two methods identified the transporter as a 45-50 kDa protein. Others have reported that not only was the glucose transporter found to be a integral part of the

plasma membrane, but it was also detected in the transverse tubule membrane (Burdett *et al.*, 1987), as well as in an undefined intracellular pool in the microsomal fractions of skeletal muscle preparations (Douen *et al.*, 1989; Wardzala and Jeanrenaud, 1981).

Several forms of glucose transporter have been recently identified (Kayano *et al.*, 1988). One form was isolated from a cDNA library of human fetal skeletal muscle, whose amino acid sequence was approximately 64% homologous with human erythrocyte glucose transporter sequences. However, studies by RNA blotting indicated that RNA transcripts encoding this unique form of skeletal muscle glucose transporter are not abundant in adult human skeletal muscle. Recently, a unique tissue-specific glucose transporter protein (Glut-4) was discovered in insulin sensitive tissues, such as adipose and skeletal muscle (James *et al.*, 1988; Fukumoto *et al.*, 1989). A monoclonal antibody (mAb 1F8) which recognised the insulin-sensitive adipose glucose transporter was used to detect an insulin-induced increase in immunolabelled glucose transporter in the plasma membrane. There was a corresponding decrease (approximately 50%) in the intracellular pool, in adipocytes (James *et al.*, 1988). There was no immunolabelling of the plasma membrane from resting adipose cells. Moreover, mAb 1F8 did not recognise the rat liver, kidney, brain, erythrocyte or HepG2 cell glucose transporter proteins (James *et al.*, 1988). James and coworkers (1989) went further and isolated cDNA from rat adipose and heart libraries. The corresponding mRNA encoded a protein that was photoaffinity-labelled with cytochalasin B and was recognised by mAb 1F8. This

cDNA was used in hybridisation studies and was found to react with mRNA from adipose, heart and skeletal muscle (James *et al.*, 1989). Therefore, an insulin-sensitive glucose transporter that was tissue-specific exists and has been suggested to possibly confer the insulin sensitivity of the tissue (James *et al.*, 1988; James *et al.*, 1989).

More recently the mAb 1F8 has been applied to *in vivo* preparations of adipose and skeletal muscle that have been treated with insulin. In a surprising finding, instead of detecting a translocation of transporters in adipose and skeletal muscle tissues, insulin induced a rapid increase in the number of mAb 1F8 detectable glucose transporters present in the luminal plasma membrane of the capillary endothelial (Vilaro *et al.*, 1989). However, a recent study contradicted this finding (Slot *et al.*, 1990) and suggested that mAb 1F8 detectable glucose transporters are located near the cell borders of skeletal myocytes.

Recently, a discrete exercise-regulatable glucose transporter has been reported to exist in rat skeletal muscle (Douen *et al.*, 1989; 1990).

1.4 MECHANISM OF INSULIN ACTION ON GLUCOSE TRANSPORT IN SKELETAL MUSCLE

An initial and major effect of insulin on skeletal muscle is to stimulate glucose transport. The mechanism of insulin action on glucose transport in skeletal muscle has been shown to be similar to that described in adipose tissue. The action on adipose tissue is well documented elsewhere (Simpson and Cushman, 1986; Kahn and Cushman, 1985). Kinetic studies have shown that in adipose tissue the effect of insulin on glucose transport is attributable to an increase in the maximal transport rate (V_{max}). There is no apparent change in glucose transporter affinity (K_m) for glucose (Simpson and Cushman, 1986; Okuno and Gliemann, 1987; Suzuki and Kono, 1980). Some authors have reported that insulin stimulates glucose transport by lowering the glucose transporter K_m (Toyoda *et al.*, 1987; Whitesell and Abumrad, 1985). However, the major body of evidence supports the earlier process. Two mechanisms can be postulated from these findings. A change in the V_{max} may be due to: (i) a change in the intrinsic activity of glucose transporters that already exist in the plasma membrane, and/or (ii) an increase in plasma membrane transporters, which could be due to either recruitment of pre-existing transporters or new transporter synthesis. Both mechanisms will increase the number of functional transporters in the plasma membrane. The latter mechanism is generally accepted by many authors (Simpson and Cushman, 1986), although a growing number of reports suggest that translocation alone does not account for the full increase in insulin-stimulated glucose transport (Baly and Horuk, 1988).

Both of the above mechanisms may play an important role in the action of insulin in adipose tissue (Calderhead and Lienhard, 1988; Carter-Su and Czech, 1980; Gibbs *et al.*, 1988).

Kinetic studies reveal that insulin stimulates glucose transport in skeletal muscle by increasing V_{max} (Rennie *et al.*, 1983) without any apparent change in the K_m (Nesher *et al.*, 1985; Idstrom *et al.*, 1986; Sternlicht *et al.*, 1988, 1989). Insulin treatment resulted in an increase in the number of glucose transporters in the plasma membrane. This was associated with a corresponding decrease in the number of glucose transporters in the microsomal fraction of isolated rat diaphragm (Wardzala and Jeanrenaud, 1981, 1983), rat hindlimb (Klip *et al.*, 1987), and L6 muscle cells (Ramlal *et al.*, 1988).

It is unclear whether translocation accounts fully for the changes in glucose transport. One report, which used L6 muscle cells, demonstrated that the increase in glucose transport caused by insulin corresponded solely to the increased translocation of transporter to the plasma membrane (Ramlal *et al.*, 1988). Moreover, another study reported that in rat hindlimb muscles the 3.2-fold increase in glucose transport could not be fully accounted for by the 1.7-fold increase in the number of glucose transporter molecules (Sternlicht *et al.*, 1988). Thus, the insulin induced changes in skeletal muscle glucose transport are similar to the effect of insulin on adipose tissue. A combination of translocation and/or changes in transporter intrinsic activity may be involved.

1.5 MECHANISM OF CONTRACTION-INDUCED INCREASE IN GLUCOSE TRANSPORT IN SKELETAL MUSCLE

Until recently, muscle contraction induced glucose uptake was considered to require the presence of a "permissive" amount of insulin (Berger *et al.*, 1975). However, over the last decade evidence has accumulated that opposes this view and suggests that the effects of exercise and insulin on muscle glucose transport are independent.

Most of the strategies used to show the independence of contraction-induced glucose uptake from insulin have been by the removal of circulating insulin from the system. Two approaches have been used to reduce plasma insulin levels below detectable limits. They include the use of anti-insulin serum or streptozotocin treated animals. Streptozotocin functions very specifically to destroy the insulin producing B-cells of the Islets of Langerhan in the pancreas. Using the latter approach, Wallberg-Henriksson and Holloszy (1984), demonstrated a 6-fold increase in glucose uptake during contraction in perfused hindlimb preparations. In a similar preparation, Ploug and co-workers (1984) found a 5-fold increase in glucose uptake during contraction when both the streptozotocin and anti-insulin serum were used to eliminate insulin from the system. Similar findings were also demonstrated by Idstrom and co-workers (1986) who found a 4-fold increase in glucose uptake during contraction in streptozotocin treated animals. The independence of contraction-induced glucose uptake from insulin was further emphasized by Wallberg-Henriksson and Holloszy (1985) and Nesher and co-workers

(1985) using isolated epitrochlearis muscle preparations from rats pretreated with streptozotocin and anti-insulin serum. These muscles were washed extensively before stimulation, which resulted in approximately 5×10^{17} -fold dilution of the extracellular space (Wallberg-Henriksson, 1987). Muscle contraction still resulted in a 3- and 2-fold increase in glucose uptake respectively. Therefore, insulin does not play a role in exercise-induced glucose transport in skeletal muscle.

However, the mechanism by which exercise stimulates glucose transport may be similar to that of insulin. Contraction of muscle has an insulin-like effect on glucose uptake which can be observed in response to both normal exercise and to electrical stimulation *in situ* or *in vivo* (Holloszy *et al.*, 1986). Like insulin, contractile activity can increase the rate of glucose transport by increasing the transporter V_{max} without any change in its K_m (Narahara and Ozand, 1963; Nesher *et al.*, 1985; Idstrom *et al.*, 1986; Sternlicht *et al.*, 1989). Two recent reports have demonstrated an increase in the number of plasma membrane glucose transporters (determined by cytochalasin B binding) in exercised skeletal muscle when compared to resting muscle (Hirshman *et al.*, 1988; Douen *et al.*, 1989). However, both groups of authors were unable to find a concomitant decrease in the number of cytochalasin B binding transporter proteins in the intracellular membrane fraction. Therefore, the lack of detection of a decreased intracellular pool of glucose transporter protein upon exercise may suggest that either a second recruitable transporter pool exists (Douen *et al.*, 1989) or perhaps that

reserve transporters are already associated with the plasma membrane and are present in an inactive state or in a masked form that does not bind cytochalasin B readily. Muscle contraction may produce a conformational change, thereby increasing glucose transporter activity and cytochalasin B binding (Hirshman *et al.*, 1988).

Hirshman and co-workers (1988) and Douen and co-workers (1989) both discovered the exercise-induced increase in glucose uptake was not solely accounted for by the increase numbers of glucose transporters in the plasma membrane. Both laboratories reported a 3- to 5-fold increase in glucose transport but only a 2-fold increase in plasma membrane associated glucose transporters. This discrepancy suggests an additional factor other than transporter recruitment was necessary.

Furthermore, a recent report showed that sarcolemmal vesicles isolated from exercised animals demonstrated no change in the number of cytochalasin B binding sites compared with that found in sarcolemmal vesicles isolated from resting muscle. However there was an increase in the V_{max} without any change in K_m (Sternlicht *et al.*, 1989). These authors concluded that the exercise-induced increase in glucose uptake was solely due to an increased turnover rate by existing transporter molecules and exercise and insulin act by different mechanisms to stimulate glucose transport in skeletal muscle, an opinion shared by others (Nesher *et al.*, 1985).

The mechanism of exercise-induced glucose uptake is still an open question. Evidence is accumulating which suggest a translocation of glucose transporters together with an increase in the intrinsic activity of

transporters already present in the plasma membrane are responsible for the observed changes in glucose transport. A number of factors may cause these changes.

1.6 POTENTIAL REGULATORS OF CONTRACTION-INDUCED GLUCOSE UPTAKE IN SKELETAL MUSCLE

A number of potential regulators of muscle glucose transport are summarised in Table 1.1. Due to their ability to influence glucose transport, some of these regulators have been suggested to mediate contraction-induced glucose transport.

Hypoxia is a potent stimulator of glucose uptake into skeletal muscle (Idstrom *et al.*, 1986). However, it is unlikely to be the sole regulator of contraction-induced glucose transport because during light exercise where hypoxia would be minimal, an increase in glucose transport was demonstrated (Katz *et al.*, 1986). During intense exercise however, hypoxia may enhance glucose transport possible through changes in the energy state of the muscle cells (e.g. ATP levels) and the production of purine breakdown products. But adenosine, the expected breakdown product from ATP during muscle hypoxia (Meghji *et al.*, 1988) has been demonstrated by means of indirect methods to inhibit glucose transport in skeletal muscle. Isolated soleus muscles were treated with adenosine deaminase (an enzyme that removes adenosine by converting it to inosine) or the adenosine-receptor antagonist 8-phenyltheophylline.

TABLE 1.1 Potential acute regulators of the glucose transport system in skeletal muscle

Groups	Stimulation	Inhibition
Hormones	Insulin	Catecholamines
Substrate	Glucose Concentration	
Oxygen	Hypoxia	
Other factors	Increased blood flow	
Activity state	Concentration	
Energy state	ATP, PCr	
Other factors	Ca ²⁺	

(Adapted from Wallberg-Henriksson, 1987).

Both treatments increased the insulin sensitivity of the glucose transport mechanism (Budohoski *et al.*, 1984; Challiss *et al.*, 1984). Therefore, even during intense exercise hypoxia is not a likely stimulator of contraction-induced glucose transport.

The energy state of muscle reflected by the ATP/ADP and PCr/Cr ratios would be an obvious regulator of glucose transport, as a decrease in these ratios will lead to an increase in glycolysis. A subsequent increase in glucose transport will follow in order to maintain glycolysis. Walker and co-workers (1982) found that in the perfused rat hindlimb, the rate of contraction induced glucose uptake was related to the ATP/ADP ratio and the PCr content of the muscle. Furthermore, in humans, Katz and co-workers (1986) demonstrated a correlation between muscle glucose uptake and PCr levels during bicycle exercise. However, the coupling between the energy state of the muscle cell and glucose uptake remains unknown.

Muscle contraction is associated with increased blood flow. Grubb and Snarr (1977) were the first to demonstrate that an increased blood flow in resting muscle leads to an enhanced glucose uptake. A subsequent explanation for contraction-induced glucose uptake was the increase in blood flow of the exercising muscle resulted in a increase in the availability of glucose and insulin. The result is an increase rate of glucose transport (Garratt *et al.*, 1972; Vranic *et al.*, 1976). However, it has since been demonstrated with *in vitro* studies that an increase in glucose uptake due to muscle contraction can occur without any change in blood flow (Wallberg-Henriksson, 1987). Thus, blood flow together

with the other regulatory factors described above are unsatisfactory in explaining the mechanism of contraction-induced glucose transport.

Agents that stimulate glucose transport, such as electrically- or chemically-induced muscle contraction also elevate cytosolic Ca^{2+} concentrations. Intracellular Ca^{2+} may mediate contraction-induced glucose uptake (Klip, 1984; Holloszy and Narahara, 1965; Clausen *et al.*, 1975). However, controversy exists in the literature concerning the apparent role of Ca^{2+} in glucose transport (Klip, 1984; Clausen, 1980). In skeletal muscle, the role of Ca^{2+} are further complicated by the fact that only transient rises in intracellular Ca^{2+} are associated with the contraction process. The Ca^{2+} concentration immediately returns to basal level after each contraction, whereas the rate of glucose transport remains elevated (Ivy and Holloszy, 1981). The evidence to date is unclear as to whether Ca^{2+} has a direct role in glucose uptake. However, there is probably another component involved as suggested by recent findings that excitation-contraction coupling involves the hydrolysis of phosphoinositols which release inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Vergara *et al.*, 1985). IP_3 has been shown to cause the release of Ca^{2+} from intracellular store (Volpe *et al.*, 1985). DAG is an activator of PKc (Nishizuka, 1984). A possible substrate for PKc has been shown to be the glucose transporter (Witters *et al.*, 1985). Therefore the link between Ca^{2+} and glucose transport involving PKc is suggested.

1.7 EXCITATION-CONTRACTION COUPLING IN SKELETAL MUSCLE

Activation of skeletal muscle contraction under physiological conditions involves depolarisation of transverse tubular regions of the sarcolemma (Huxley and Taylor, 1958; Hodgkin and Horowicz, 1960; Costantin and Taylor, 1973; Costantin, 1975) leading to Ca^{2+} release from the terminal cisternae of the sarcoplasmic reticulum (Stephenson, 1981). The transverse tubular-terminal cisternae transmembrane signaling mechanism apparently is non-electrical (Donaldson, 1985; Donaldson *et al.*, 1987 and references therein) and there have been numerous studies aimed at examining the chemical triggering of sarcoplasmic reticulum Ca^{2+} release. In this regard some researchers have examined the role of inositol phosphates in the mobilisation of intracellular skeletal muscle Ca^{2+} (Volpe *et al.*, 1985; Vergara *et al.*, 1985; Thieleczek and Heilmeyer, 1986; Nosek *et al.*, 1986; Donaldson *et al.*, 1987). Such studies were based on findings where IP_3 elicited Ca^{2+} release from the endoplasmic reticulum of a wide variety of cells (Berridge, 1981; Suematsu *et al.*, 1984; Hirata *et al.*, 1984; Hokin, 1985).

Volpe and co-workers (1985) were the first to demonstrate IP_3 as an agent which can cause the release of Ca^{2+} from isolated, purified sarcoplasmic reticulum. Furthermore, they demonstrated that IP_3 could elicit isometric force development in chemically skinned muscle fibres (Volpe *et al.*, 1985). Others have similarly found that IP_3 could induce Ca^{2+} release (Donaldson, 1985; Donaldson *et al.*, 1987; Vergara *et al.*, 1985) and isometric force production (Theileczek and Heilmeyer *et al.*,

1986; Vergara *et al.*, 1985) in skinned skeletal muscles fibres. However, there is some controversy as to whether IP_3 could be the chemical transmitter for excitation-contraction in skeletal muscle (Volpe *et al.*, 1987). A number of authors have been unable to confirm that IP_3 can induce Ca^{2+} release from sarcoplasmic reticulum of skeletal muscle (Volpe *et al.*, 1987; Mikos and Snow, 1987; Somlyo *et al.*, 1988).

Although the role of IP_3 in excitation-contraction coupling of skeletal muscle is plausible if not consolidated, the mechanism by which skeletal muscle depolarisation generates IP_3 is of considerable interest. Vergara and co-workers (1985) demonstrated that IP_3 was generated by electrical stimulation of skeletal muscle. IP_3 was able to release Ca^{2+} from sarcoplasmic reticulum in skinned muscle fibres. Their findings lead to the hypothesis for the proposed role of IP_3 as a chemical link in excitation-contraction coupling (Fig. 1.2). T-tubular membrane depolarisation stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI4,5-bisP) by a phosphatidylinositol-specific phospholipase. The free IP_3 is water soluble and can diffuse to the sarcoplasmic reticulum to activate Ca^{2+} release by stimulating the Ca^{2+} channels to open. IP_3 is rapidly degraded by a specific IP_3 phosphatase (Milani *et al.*, 1988), via various routes of metabolism into a series of intermediates which are eventually converted to inositol (Berridge and Irvine, 1989).

The second product of PI4,5-bisP hydrolysis is DAG and is a potent activator of PKC (Nishizuka, 1984). Evidence has accumulated suggesting PKC has an important function in the stimulation of glucose transport.

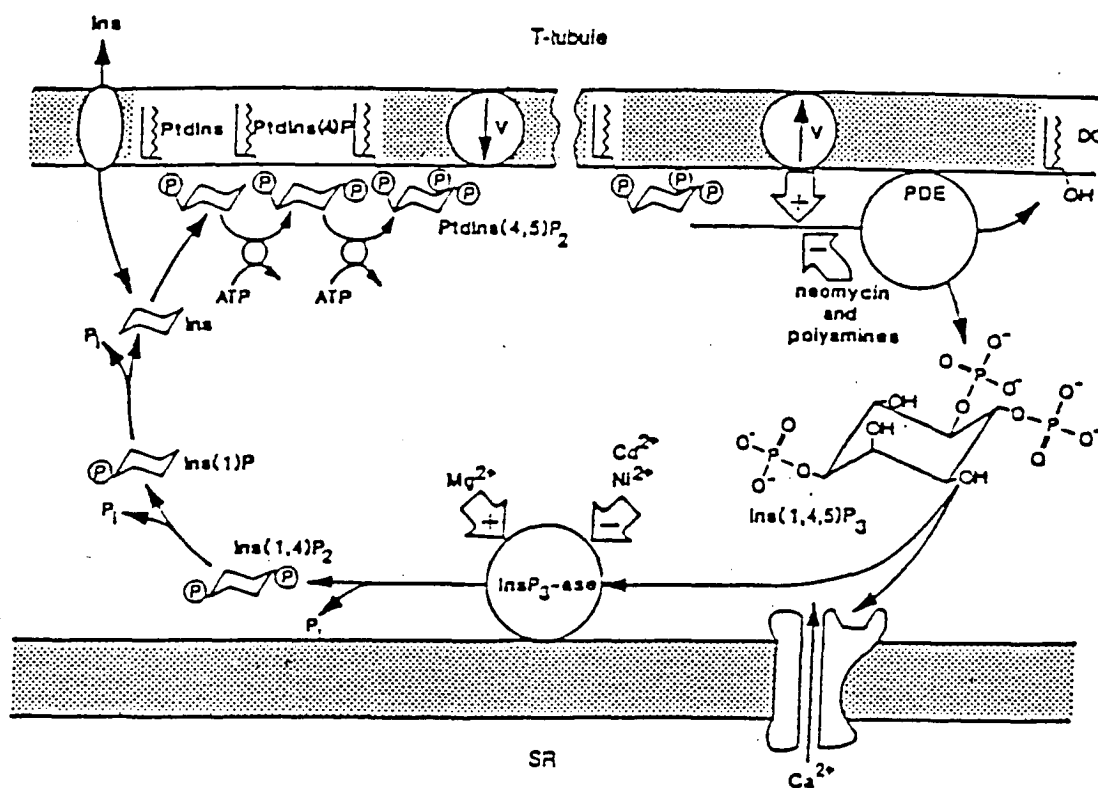


FIG. 1.2. THE PROPOSED ROLE OF INOSITOL 1,4,5-TRISPHOSPHATE AS A CHEMICAL LINK IN EXCITATION-CONTRACTION COUPLING (from Vergara *et al.*, 1985)

1.8 THE PROPERTIES OF PKc

The protease-activated form of PKc was first found in the soluble fraction of rat brain (Takai *et al.*, 1977). It was later found that phospholipid and Ca^{2+} were potent activators of PKc (Takai *et al.*, 1979). PKc is a single polypeptide with an apparent molecular weight between 77-87 kDa depending on the method of determination (Turner and Kuo, 1985; Farooqui *et al.*, 1988). The enzyme is divided into two functional domains. A hydrophobic regulatory subunit that can insert into the membrane and a hydrophilic catalytic subunit (Kikkawa *et al.*, 1983). PKc can be found in a wide variety of tissue types. The largest amount of PKc activity has been found in brain, spleen and platelets. Skeletal and heart muscle contains the least amount of the enzyme when expressed per mg protein (Kuo *et al.*, 1980; Minakuchi *et al.*, 1981).

Despite attempts to determine the intracellular topography of PKc, its subcellular distribution still remains uncertain. This is mostly due to enzyme extraction procedures that require the presence of Ca^{2+} chelators to prevent Ca^{2+} -dependent proteolysis of PKc by calpains (Nishizuka, 1986). Immunological studies, however, using monoclonal antibodies against PKc indicate that the subcellular distribution of PKc varies in the different cell types (Nishizuka, 1986; Kikkawa and Nishizuka, 1986).

PKc was first found to be fully enzymatically active when Ca^{2+} and a "membrane-associated factor" were simultaneously present (Takai *et al.*, 1979b). The "membrane-associated factor" was subsequently

identified as a phospholipid. Phosphatidylserine was found to be the most effective activator of PKc and is now commonly used in most *in vitro* PKc assays. Phosphatidylinositol and phosphatidylethanolamine alone were less effective whereas phosphatidylcholine, phosphatidic acid and sphingomyelin alone were ineffective (Takai *et al.*, 1979; Kaibuchi *et al.*, 1981). However, recent studies have shown that short chained neutral phosphatidylcholines, alone, were able to activate PKc *in vitro* (Walker and Sando, 1988). This opens an interesting area as to the nature of the fatty acid acyl group of the PKc activating phospholipids. Moreover, the combination of these phospholipids show a positive and a negative co-operativity in the activation of PKc. Enzymatic activity was enhanced when phosphatidylethanolamine was added together with phosphatidylserine. Conversely, activity was inhibited upon the addition of phosphatidylcholine or sphingomyelin together with phosphatidylserine (Kaibuchi *et al.*, 1981). It is possible that co-operative interaction of these phospholipids in the lipid bilayer may act to regulate PKc activity.

DAG can stimulate PKc activity by increasing the enzyme's affinity for both Ca^{2+} and phospholipid (Kishimoto *et al.*, 1980). DAG binds to the regulatory domain of PKc and allosterically alters the enzyme. DAG can effectively reduce the Ca^{2+} requirement for PKc activation such that the enzyme is fully activated without any change in cell Ca^{2+} concentration (Takai *et al.*, 1979; Kishimoto *et al.*, 1980). A mixed micelle model has been used to investigate the stoichiometry of PKc activation by Ca^{2+} , DAG and phospholipids (Hannun *et al.*, 1985, 1986). Hannun and co-workers (1986) have proposed that the activation of a

monomeric PKc requires a highly ordered complex consisting of four molecules of phosphatidylserine and one molecule of Ca^{2+} that binds to the four carbonyl groups present in the four phosphatidylserine head groups. The inactive monomeric PKc protein binds to this complex. DAG binds to the PKc-phosphatidylserine- Ca^{2+} complex via the two carbonyls and the three hydroxyl groups of DAG (Ganong *et al.*, 1986).

The stereospecificity and fatty acyl moieties of DAG required to activate PKc have been extensively studied. In both the intact and cell free preparations only the natural 1,2-sn-DAG configuration was active (Rando and Young, 1984; Nomura *et al.*, 1986). Furthermore, at least one unsaturated fatty acid at either position 1 or 2 can fully stimulate PKc activity *in vitro* irrespective of fatty acid chain length (Mori *et al.*, 1982).

1.9 PHORBOL ESTERS

Phorbol esters are tumour promoters that were first discovered in the oil of *Euphorbiacea croton tiglium* L. They are lipophilic, therefore are able to diffuse readily across plasma membranes. The most potent phorbol ester is 12-O-tetradecanoyl-phorbol-13-acetate (TPA). This molecule has a DAG-like structure and binds specifically to PKc molecules *in vivo* and *in vitro* (Ashendel, 1985). TPA mimics the action of DAG by increasing the affinity of PKc for Ca^{2+} and phospholipid (Nishizuka, 1984) and is a commonly used tool to study the activation and subsequent function of PKc.

1.10 PHYSIOLOGICAL ACTIVATION OF PKc

There are two mechanisms that can cause PKc activation. The first is by translocation of the enzyme to the membrane where the enzyme can come into close proximity to membrane bound phospholipids, DAG or phorbol esters. A variety of cell types exhibit PKc translocation in response to phorbol ester, hormonal or other agonist treatment (Niedel and Blackshear, 1986). However the actual mechanical process of this phenomenon remains unclear. Ca^{2+} mobilisation is suggested to trigger PKc translocation (Trilivas and Heller Brown, 1989; May *et al.*, 1985; TerBush *et al.*, 1988). In adrenal chromaffin cells the effect of K^{+} induced depolarisation was a rapid and transient increase in PKc activity isolated from membranes (TerBush *et al.*, 1988). In astrocytoma cells the translocation of PKc into membranes was caused by the Ca^{2+} ionophore, ionomycin (Trilivas and Heller Brown, 1989). Phenylephrine induced translocation of PKc in rat pinealocytes was blocked by treatment with EGTA or inorganic Ca^{2+} blocker. In addition, the Ca^{2+} ionophore A23187 was able to trigger PKc translocation (Ho *et al.*, 1988). Therefore, it is possible that agonist induced increase in intracellular Ca^{2+} concentration can trigger the recruitment of PKc into the membrane and "prime" the enzyme for activation by transient increase in DAG (May *et al.*, 1985).

The second mechanism where PKc can be transformed into an activated state is by proteolytic cleavage. PKc, particularly membrane bound enzyme, can be cleaved by a Ca^{2+} -dependent protease (Calpain)

into its respective regulatory and catalytic fragments. This latter fragment is called protein kinase M (PKm). PKm is hydrophilic and thus able to travel freely inside the cell to target proteins that are not in the immediate vicinity of the plasma membrane. Moreover, PKm differs from PKc by having a phospholipid- and Ca^{2+} -independent kinase activity (Murray *et al.*, 1987). However, PKm formation has only been reported in platelets (Tapley and Murray, 1984) and neutrophils (Melloni *et al.*, 1985). Therefore, this mechanism of PKc activation could be tissue specific. Indeed, the treatment of bovine adrenal cortex with ACTH resulted in PKc translocation, but there was no PKm formation (Vilgrain *et al.*, 1984).

1.11 PKc ISOZYMES

Since the discovery of two bovine and human genes that encoded polypeptide sequences that were highly homologous with PKc (Coussens *et al.*, 1986), there have been several PKc isozymes identified (Nishizuka, 1988; Kikkawa *et al.*, 1989). The isozymes can be divided into two major groups dependent on the type of conserved C2 region (Soderling, 1990). However, all the isozymes contain a highly conserved kinase catalytic domain. Studies to determine the tissue distribution of the PKc isozymes have revealed a pattern of tissue specific expression. The PKc isozyme type III is found solely in the brain and in the spinal cord. The PKc type II is found in brain, pituitary cells, pancreatic islets and skeletal muscle.

The PKc type I isozyme has a wide distribution and can be found in many cell types (Kikkawa *et al.*, 1989). Moreover, a recent study has demonstrated that the expression of PKc isozymes can be controlled differently during the various stages of development (Yoshida *et al.*, 1988). Thus, the isozymes may have different functions with respect to the transduction of signals and substrate specificity. In fact, studies using isolated PKc isozymes I,II and III have shown that they differ in their modes of activation and kinetic properties (Sekiguchi *et al.*, 1988). Therefore, PKc isozymes provide a mechanism where the diverse range of cellular processes that seem to involve PKc can be explained.

1.12 "DOWN-REGULATION" OF PKc BY PHORBOL ESTERS

Prolonged exposure of tissues to phorbol esters can lead to the loss of PKc activity from the cells. This phenomenon is called "down-regulation" and is specific to active phorbol esters (i.e. those which activate PKc), such as, TPA (Niedel and Blackshear, 1986). The process of PKc down-regulation is attributed to an increased rate of enzyme degradation. There is no change in the rates of either enzyme synthesis or mRNA transcription (Young *et al.*, 1987). TPA is not readily metabolised, thus, TPA can hold PKc molecules in the membrane for a prolonged period. This extended association of PKc with the plasma membrane is thought to be sufficient to cause enzyme proteolysis (Kikkawa and Nishizuka, 1986). PKc down-regulation is commonly used

to investigate the importance of PKc dependent pathways in the actions of a variety of stimuli (Niedel and Blackshear, 1986).

1.13 SUBSTRATE SPECIFICITY OF PKc

To fully appreciate the diverse range of influences PKc has on cellular responses, a knowledge of enzyme substrates is essential (Nishizuka, 1986). PKc phosphorylates seryl and threonyl residues but not tyrosyl residues in proteins (Nishizuka, 1986). It has a broad range of substrates of which some are listed in Table 1.3. Common to many kinases, PKc can undergo autophosphorylation (Huang *et al.*, 1986). One important protein that has been demonstrated to be a substrate for PKc has been the glucose transporter (Witters *et al.*, 1985; Gibbs *et al.*, 1986; Joost *et al.*, 1987). Therefore, PKc may be involved in the regulation of the glucose transporter activity. There is considerable evidence that supports this hypothesis.

1.14 PKc AND GLUCOSE TRANSPORT

TPA can stimulate glucose transport in a variety of cell types. A summary of these studies is listed in Table 1.4. TPA binds to the DAG binding site on the regulatory domain of the enzyme (Ashendel, 1985) and mimics DAG and activates the enzyme (Nishizuka, 1984). Thus, it

TABLE 1.3 **PKc Substrates**

<u>Receptor proteins</u>	<u>Enzymes and enzyme-related proteins</u>
Epidermal growth factor receptor	Glycogen phosphorylase kinase
Insulin receptor	Glycogen synthase
Somatomedin C receptor	Phosphofructokinase
Transferrin receptor	Tyrosine hydroxylase
Interleukin-2 receptor	NADPH oxidase
Nicotinic acetylcholine receptor	Cytochrome P450
Immunoglobulin E receptor	Guanylate cyclase
Retinoid-binding protein	DNA methylase
β -Adrenengic receptors	Phospholamban
	Myosin light chain kinase
	Initiation factor 2
	Ribosomal S6 protein
	Adenylate cyclase
	Phospholipase C
	Ca^{2+} -ATPase
<u>Membrane proteins</u>	<u>Nuclear proteins</u>
Ca^{2+} -transport ATPase	Histone
$\text{Na}^{+}/\text{K}^{+}$ ATPase	Protamine
Na^{+} channel protection	High-mobility-group protein
$\text{Na}^{+}/\text{H}^{+}$ exchange system	
Glucose transporter	
GTP-binding protein	
HLA antigen	
Synaptic B50 (F1) protein	
<u>Contractile and cytoskeletal proteins</u>	<u>Other proteins</u>
Myosin light chain	pp60src protein
Troponin T and I	Fibrinogen
Vinculin	GABA modulin
Filamin	Stress proteins
Caldesmon	Myelin basic protein
Cardiac C-protein	Middle T antigen
Microtubule-associated proteins	Class I HLA antigens

(Adapted from Nishizuha, 1986; Takai *et al.*, 1985; Farooqui *et al.*, 1988)

(Note: Only some of the substrates listed above have been)
conclusively shown to be substrates in vivo)

TABLE 1.4 **Effect of phorbol esters on glucose transport**

Cell type	Phorbol ester	Concentration	Time for effect	Effect on glucose transport
Rat adipocyte	PMA	1 nM	4 sec	Incr. 3x
Rat adipocyte	DAG	0.1 mM	1 hr	Incr. 3–5x
Rat thymocyte	PMA	0.16 μ M	0.3–1 hr	Incr. 3x
Mouse thymocyte	PMA	0.16 μ M	0.3–1 hr	Incr. 3x
Rat sk muscle	PMA/OAG	0.8 μ M	0.5–1 hr	No Incr.
Human neutrophil	PMA/OAG	1 nM/33 μ M	1 hr	Incr. 3 x, 6x
Human fibroblast	PMA	0.3 μ M	0.3 hr	Incr. 0.5x
BALB/c3T3 preadipocyte	PMA	0.16 μ M	4 hr	Incr. 2x17x
CE fibroblast	PMA	0.1 μ M	4 hr	Incr. 3–4x
3T3 fibroblast	MOG	0.1 mM	1 hr	Incr. 0.2x
3T3 fibroblast	PMA	0.1 μ M	3 hr	
3T3-L1 fibroblast	PDB	1 μ M	0.5 hr	Incr. 1x
3T3-L1 adipocyte	PDB	1 μ M	0.5 hr	Incr. 1.3x
3T3-L1 adipocyte	PMA	1 μ M	0.3–1 hr	Incr. 2x
HeLa cell	PMA	1–40 nM	0.5–1 hr	Incr. 2x
L6 muscle cell	PDB	0.1 μ M	0.6 hr	Incr. 0.5x
BC3H-1 myocyte	PMA	0.1 μ M	0.2 hr	Incr. 4x
BC3H-1	OAG	0.25 mM	0.2 hr	Incr. 0.4x
Human epidermal cells	PMA	16 nM	3 hr	Decr. 0.3x

Incr.: increase; Decr.: decrease; CE: chick embryo; PMA: phorbol myristate acetate; OAG: 1–O– octadecenyl, 2–O– acetyl glycerol; MOG: 1–Mono oleyl glycerol; PDB: 4 β –phorbol–12, 13–dibutyrate; sk: skeletal.
(Adapted from Klip and Douen, 1989)

can be postulated that PKc participates directly in the stimulatory action TPA has on glucose transport. Initial studies looked at the level of glucose transporter phosphorylation in response to TPA treatment. In studies where antibodies were used against the human erythrocyte glucose transporter to immunoprecipitate the transporter, it was found that phorbol esters stimulated the phosphorylation of the glucose transporter in human erythrocytes (Witters *et al.*, 1985), in 3T3-L1 adipocytes (Gibbs *et al.*, 1986) and in isolated rat adipose cells (Joost *et al.*, 1987). In addition, isolated PKc was shown to phosphorylate the glucose transporter protein *in vitro* (Witters *et al.*, 1985). However, no correlation was found in any of these studies between the degree of transporter phosphorylation and changes in glucose transport activity. However, these findings must be considered in light of the recent discovery of an insulin-regulatable glucose transporter protein (James *et al.*, 1988). It could be possible that the antibody against the human erythrocyte glucose transporter may only recognise one subtype of transporter. Thus, it remains uncertain whether transporter phosphorylation is linked to transporter activation.

As previously mentioned (section 1.5) glucose transporter activation could involve both translocation and changes in intrinsic activity of the transporter. Several studies demonstrated that phorbol esters can cause transporter translocation. TPA treatment of Swiss 3T3 fibroblast cells resulted in a decrease in cytochalasin B binding to microsomal sites and a corresponding increase in the number of binding sites on the plasma membrane (Kitagawa *et al.*, 1985). The effect of TPA

was not diminished by treatment with cycloheximide which suggested a protein synthesis independent translocation of a pre-existing stored transporter. A more detailed study was undertaken by Muhlbacher and co-workers (1988) to demonstrate the mechanism whereby TPA stimulated glucose transport. TPA treatment of rat adipose cells resulted in a translocation of glucose transporters and was concurrent with a similar fold increase in the glucose transport rate (Muhlbacher *et al.*, 1988). Moreover, kinetic studies revealed that TPA-stimulated glucose transport in BC3H-1 myocytes was due to an increase in V_{max} with no change in K_m of the glucose transporter (Standaert *et al.*, 1988). Although brief exposures of phorbol esters can be related to glucose transporter translocation, the precise role of PKC in the translocation event is obscure.

Prolonged exposure of cells to phorbol esters can result in enhanced synthesis of glucose transporter mRNA. The treatment of FR3T3 cells with TPA for 18 hr resulted in an increase quantity of transporter mRNA that was similar to the 3-5 fold corresponding increase in glucose transport activity (Flier *et al.*, 1987). Short TPA exposures did not lead to any changes in mRNA levels.

An alternative approach to determine if PKC was implicated in glucose transport was to introduce exogenous phospholipase C to cells in an attempt to enhance phosphoinositol hydrolysis and increase the level of DAG. In rat adipose cells (Goldman and George, 1988) and BC3H-1 myocytes (Standaert *et al.*, 1988) exogenous phospholipase C stimulated glucose transport.

The ability of phorbol esters to mimic the action of insulin has implicated PKc as a possible intracellular effector for insulin. Recent published data has tended to support this possibility. In BC3H-1 myocytes, insulin treatment was found to increase levels of DAG (Saltiel *et al.*, 1987; Farese *et al.*, 1987). The source of this DAG remains unclear, as it was shown to arise from *de novo* synthesis (Farese *et al.*, 1987) as well as from hydrolysis by a specific phospholipase C of a novel insulin-sensitive glycosyl-phosphatidylinositol (Saltiel *et al.*, 1988). Furthermore, in the same cell type insulin treatment also resulted in an increase in plasma membrane associated PKc activity. An increase in cytosolic PKc activity was also observed (Cooper *et al.*, 1987). More detailed analysis by the same laboratory using PKc antibodies demonstrated a transient rise in plasma membrane PKc activity which corresponded with a decrease in cytosolic PKc activity (Acevedo-Duncan *et al.*, 1989). Both DAG production and PKc translocation is the primary mechanism for PKc activation (Nishizuka, 1984). However, the insulin effect on DAG levels and PKc translocation in BC3H-1 myocytes seems to be specific for this cell type. Other studies using 3T3-L1 fibroblasts (Klip *et al.*, 1988), 3T3-L1 adipocytes (Glynn *et al.*, 1986; Klip *et al.*, 1988) and L6 muscle cells (Klip and Ramlal, 1987) do not show any of these insulin induced changes to PKc distribution nor DAG production. However, TPA treatment of these cells can cause PKc translocation and stimulation of glucose transport. Therefore, due to the differing effects of TPA and insulin these compounds may act by differing mechanisms to influence glucose transport.

Two other approaches used to investigate the role of PKc in insulin-induced glucose transporter activation are: (i) addition of PKc inhibitors and (ii) down-regulation of PKc. In rat adipocytes the PKc inhibitor, sphingosine, was able to inhibit the effect of insulin on glucose transport, but had no effect on the basal transport rates (Robertson *et al.*, 1989). This suggests that PKc is not involved in the maintenance of basal levels of glucose transport.

In contrast, the effect of insulin was not inhibited by prior down-regulation of PKc from L6 skeletal muscle cell (Klip and Ramlal, 1987) or BC3H-1 myocytes (Cooper *et al.*, 1989). However, before any conclusion can be made, it should be noted that TPA induced-down-regulation of PKc was not completely effective in the BC3H-1 myocytes. The PKc type II isozyme was shown to remain after the TPA treatment and may explain the continued effectiveness of insulin.

1.15 IS CONTRACTION-INDUCED GLUCOSE TRANSPORT IN SKELETAL MUSCLE MEDIATED BY PKc?

As already illustrated there are many similarities between the glucose transport response to both insulin and contraction in skeletal muscle. Both stimuli can cause increased glucose transport by inducing either transporter translocation and/or increased transporter intrinsic activity. There is some evidence that PKc may play an integral part in these mechanisms because TPA and exogenous phospholipase C are able to increase glucose transport in a variety of cells. The effect of insulin on

DAG production and PKc translocation has pointed towards PKc as a possible intracellular mediator of its glucose transport stimulatory action. The glucose transporter protein is a substrate for PKc, although no correlation between the degree of phosphorylation and changes in transporter activity have been found. Nevertheless, there is evidence that suggests that PKc may be either directly or indirectly responsible for the activation of glucose transport.

The excitation-contraction coupling hypothesis suggests that membrane depolarisation results in the production of IP₃ together with DAG. Thus, together with the transient rises in intracellular Ca²⁺, muscle contraction may lead to activation of PKc. Enzyme activation will be represented by PKc translocation and a concurrent production of DAG. As yet these events have not been studied in skeletal muscle.

The aims of the thesis are to:

- (i) investigate whether skeletal muscle contraction can regulate PKc activity;
- (ii) determine if PKc activation is correlated to contraction-induced glucose transport changes.

CHAPTER 2

PKc ACTIVITY IN RAT SKELETAL MUSCLE

2.1 INTRODUCTION

PKc has attracted exceptional interest in recent years not only because of its physiological importance as a pivotal regulatory element in signal transduction, cellular regulation and tumour promotion but also because of its novel mode of activation. PKc can be activated by a low concentration of Ca^{2+} in the presence of both phosphatidylserine and DAG. The majority of the inactive PKc form is located in the cell cytoplasm, but upon production of DAG at the inner leaflet of the plasma membrane the enzyme binds to the membrane and becomes activated (Nishizuka, 1984). Hence, *in vivo*, activation of PKc is reflected by translocation of this enzyme from the cytosol to the plasma membrane. This novel property of PKc activation has been exploited to measure its physiological state of activation.

Various methods have been employed to measure the distribution of PKc between the cytosol and the plasma membrane. These include radiolabelled phorbol ester binding (Uchida and Filburn, 1984), immunological localization *in vivo* (Girard *et al.*, 1986), and *in vitro* measurement of exogenous protein phosphorylation (Kikkawa *et al.*, 1982). The latter method is the most widely used and is preferable as it

has the ability to detect changes in both the cytosolic and particulate PKc activities.

The aim of the work described in this chapter was to develop an assay for measuring skeletal muscle PKc. Thus, the present chapter reports on the extraction, partial purification, identification and measurement of the subcellular distribution of PKc in resting skeletal muscle. A comparison is made of the distribution of PKc in relation to muscle fibre types.

2.2 MATERIALS

L- α -Phosphatidyl-L-serine, diolein, histone III-s, EGTA, trypsin and soya bean trypsin inhibitor were obtained from Sigma. [γ - 32 P]ATP (1.5×10^3 cpm/pmol) was purchased from Bresatec (Adelaide, Australia). Preswollen DE-52 cellulose and P81 Phosphocellulose paper were obtained from Whatman.

2.3 METHODS

2.3.1 REMOVAL OF THE RAT GASTROCNEMIUS-PLANTARIS-SOLEUS SKELETAL MUSCLE GROUP

Fed, male hooded Wistar rats (250 ± 10 g) were anaesthetized with pentobarbital interperitoneally (12.5 mg). The knee was secured by

the tibiopatellar ligament and the Achilles tendon was attached to a wire to hold the hindlimb in an out-stretched position. The skin was removed from the left hindlimb and the sciatic nerve exposed and cut. The gastrocnemius-plantaris-soleus muscle group was exposed by careful removal of overlaying tissue. The exposed muscle was kept moist with saline. The muscle group was rapidly removed (5 s) by attaching an arterial clamp to the distal end of the Achilles tendon. The distal end was severed and the muscle pulled upwards to expose the proximal end of the muscle group which was also subsequently severed (Fig. 2.1).

2.3.2 EXTRACTION OF PKc

The gastrocnemius-plantaris-soleus muscle group from non-exercised rats were rapidly removed (approx. 5 s) and homogenized in 4 ml ice-cold 20 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose and 1 mM DTT using an Ultra-Turrax homogenizer (2 X 30 s). The homogenate was centrifuged (1 h, 100,000 X g) and the resulting pellet (particulate fraction) extracted (1 h) with 6 ml of 20 mM Tris-HCl buffer, pH 7.5, containing 2.0% (v/v) Triton X-100, 2 mM EDTA, 10 mM EGTA and 1 mM DTT. The supernatant was collected by centrifugation (30 min, 100,000 X g). In initial studies the particulate fraction was extracted twice and the initial supernatant (cytosolic fraction) was fractionated using $(\text{NH}_4)_2\text{SO}_4$ precipitation. Omission of the second pellet extraction or $(\text{NH}_4)_2\text{SO}_4$ fraction did not significantly affect the total activity

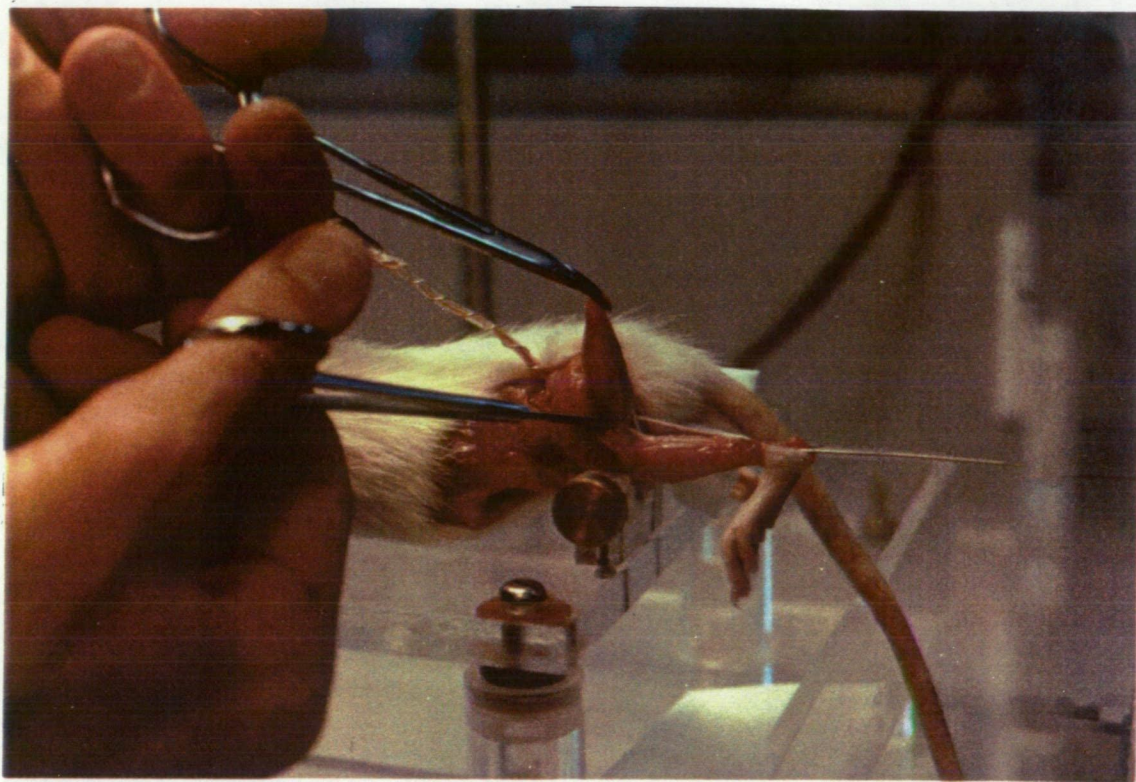


FIG. 2.1. REMOVAL OF THE GASTROCNEMIUS-PLANTARIS-SOLEUS SKELETAL MUSCLE GROUP

recovered (combined contribution <5%).

2.3.3 PARTIAL PURIFICATION OF PK_c AND PK_m BY ANION EXCHANGE CHROMATOGRAPHY

Supernatant extracts containing PK_c were routinely applied to columns (4 ml) of Whatman DE-52, which were then washed with 20 ml 20 mM Tris-HCl, pH 7.5, containing 2 mM EDTA, 0.5 mM EGTA and 1 mM DTT. PK_c and PK_m activities were eluted using linear gradients of 36 ml of 0-0.15 M and 100 ml of 0-0.4 M NaCl, respectively. Fractions of 4 ml were collected and typically PK_c activity was found in fractions 4-6 corresponding to 30-90 mM NaCl. Crude extracts were routinely applied to DE-52 columns before all PK_c determinations.

The NaCl concentration in each fraction was determined using a conductivity meter (Radiometer, Type CDM 2d).

2.3.4 PHOSPHATIDYL/DIOLEIN MICELLES PREPARATION

Phosphatidylserine/diolein vesicles were prepared as described by Kikkawa *et al.* (1982). Phosphatidylserine (3 mg) and diolein (0.06 mg) were mixed in a small volume of chloroform which was later removed by evaporation with nitrogen. The residue was resuspended in 21 ml ice-cold Tris-HCl buffer, pH 7.5 containing 0.02% sodium azide, by sonication with a Kontes sonifier (Model K881440) for 5 min on ice.

2.3.5 [γ - ^{32}P]ATP PREPARATION

ATP solution (90 $\mu\text{g}/\text{ml}$) was prepared in ice-cold 20 mM Tris-HCl buffer, pH 7.5 containing 0.02% sodium azide. The exact ATP concentration was derived from Beer's Law using a molar extinction coefficient of $15.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (Dawson *et al.*, 1962) and ATP absorbance (259 nm) measured on a Varian Cary Spectrophotometer (Model 219). A 150 μl aliquot of a 10 mCi/ml solution of (γ - ^{32}P)ATP (3000 Ci/mmol) was added to 24 ml of the "cold" ATP solution. After mixing, 1 ml aliquots were transferred into Eppendorf tubes and stored frozen (-20°C) until use. Approximate final concentration of ATP was 1477 pmol/10 μl and the specific activity at day one was $>3000 \text{ Ci}/\text{mmol}$.

2.3.6 HISTONE III-S PREPARATION

To eliminate any variation between batches of histone III-s and between different bottles of the same batch, the contents of several bottles of histone III-s were pooled. The histone III-s was dissolved in 6 ml of distilled water in a 30 ml glass centrifuge tube and lyophilized (Warburton-Franke freeze drier). A 5 mg/ml solution of lyophilized histone III-s was prepared immediately prior to use in 20 mM Tris-HCl buffer, pH 7.5.

2.3.7 PKc ASSAY

Fractions were assayed for PKc (Kikkawa *et al.*, 1982) using histone III-s as the substrate and Whatman phosphocellulose P81 paper to collect the TCA acid-precipitable material. The assay mixture contained Tris-HCl (20 mM, pH 7.5), magnesium acetate (12.5 mM), histone III-s (50 μ g), [γ - 32 P]ATP (10 μ M; specific activity $1-1.5 \times 10^3$ cpm/pmol), PKc sample (50 μ l) and either CaCl_2 (5 mM) plus phosphatidylserine (42 μ g/ml); diolein (0.84 μ g/ml) [active] or in their absence plus EGTA (2.5 mM) [basal] in a final volume of 170 μ l. The reaction was terminated after 12 min incubation at 30°C by pipetting 50 μ l aliquots to phosphocellulose paper P81 (2X2 cm) and immersing in a stirring solution of 5% TCA. The papers were washed in 5% TCA twice, water once, then dried and the radioactivity was determined by scintillation counting.

Unless otherwise indicated, PKc activity was calculated as the difference between the active and basal activities and in terms of the weight of the gastrocnemius-plantaris-soleus muscle group from non-stimulated animals (1.3 ± 0.1 g). This took into account the increase in muscle weight from blood engorgement as a result of muscle contraction. The enzyme activity was expressed as pmol of 32 P from [γ - 32 P]ATP, incorporated into histone III-s per min, per gram wet weight of muscle, at 30°C. One gram wet weight of this muscle contained 220 ± 7 (n=5) mg of protein.

2.3.8 TRYPTIC DIGESTION OF PKc

Partial proteolytic digestion of PKc to PKm was conducted essentially as described by Ferrari *et al.* (1987). Fractions containing the highest PKc activity were pooled and a 10 ml aliquot was incubated in 10 ml of Tris-HCl (20 mM; pH 8.0) buffer containing BSA (10 mg/ml) and trypsin (8 μ g/ml). The mixture was incubated at 30°C for 6 min and the reaction terminated by the addition of 1 ml of soya bean trypsin inhibitor (0.5 mg/ml) and immersion in ice. The digested samples were loaded onto Whatman DE-52 anion exchange columns and histone III-s kinase activity resolved using a 100 ml linear gradient of 0-0.5 M NaCl in 20 mM Tris-HCl, pH 7.5 containing 2 mM EDTA, 0.5 mM EGTA and 1 mM DTT.

Histone kinase activity, independent of Ca^{2+} and phospholipid, eluting at approximately 200 mM was considered as putatively PKm.

2.4 RESULTS

2.4.1 DETERMINATION OF PKc ACTIVITY IN THE RAT GASTROCNEMIUS - PLANTARIS - SOLEUS SKELETAL MUSCLE GROUP

In the course of experiments aimed at recovering PKc activity from cardiac muscle (Rattigan *et al.*, 1989) and skeletal muscle, it became apparent that routine procedures using 0.1-0.3% (v/v) Triton X-100 containing buffers (Kuo, *et al.*, 1980; Kikkawa *et al.*, 1982) were insufficient to fully extract activity associated with the particulate fraction. Thus modification of the extraction procedures was required and buffers containing a higher concentration of Triton X-100 (e.g. 2%) as well as 10 mM EGTA were found to fully extract particulate activity (extracted 95% of the particulate activity) (Table 2.1).

Three distinct peaks of histone kinase activity (I, II and III) were eluted from the DE-52 anion exchange column at approximately 10, 50 and 200 mM NaCl respectively (Fig. 2.2). The greatest histone kinase activity in the presence of Ca^{2+} , diolein and phosphatidylserine was eluted at approximately 50 mM. This is in agreement with published NaCl concentrations for the elution of PKc from this type of anion exchange column (Kikkawa *et al.*, 1982).

Peak I was only present in the cytosolic fraction. If the homogenate was treated with cAMP before anion exchange chromatography, then this peak would be absent (eluting during washout). Therefore, peak I was indicative of the undissociated form of

TABLE 2.1 Comparison of Particulate Protein Kinase C Extraction Conditions from Non-Stimulated Skeletal Muscle

Triton X100 %	EGTA (mM)	Volume ml	PKc activity (pmol/min/g muscle)		
			Cytosol	Particulate	Total
0.2	0.5	2	1088 ± 153	147 ± 28	1235 ± 177 (9)
2.0	10.0	6	1450 ± 250	2070 ± 200	3250 ± 450 (9)

Values given are means ± S.E. with the number of animals shown in parenthesis.

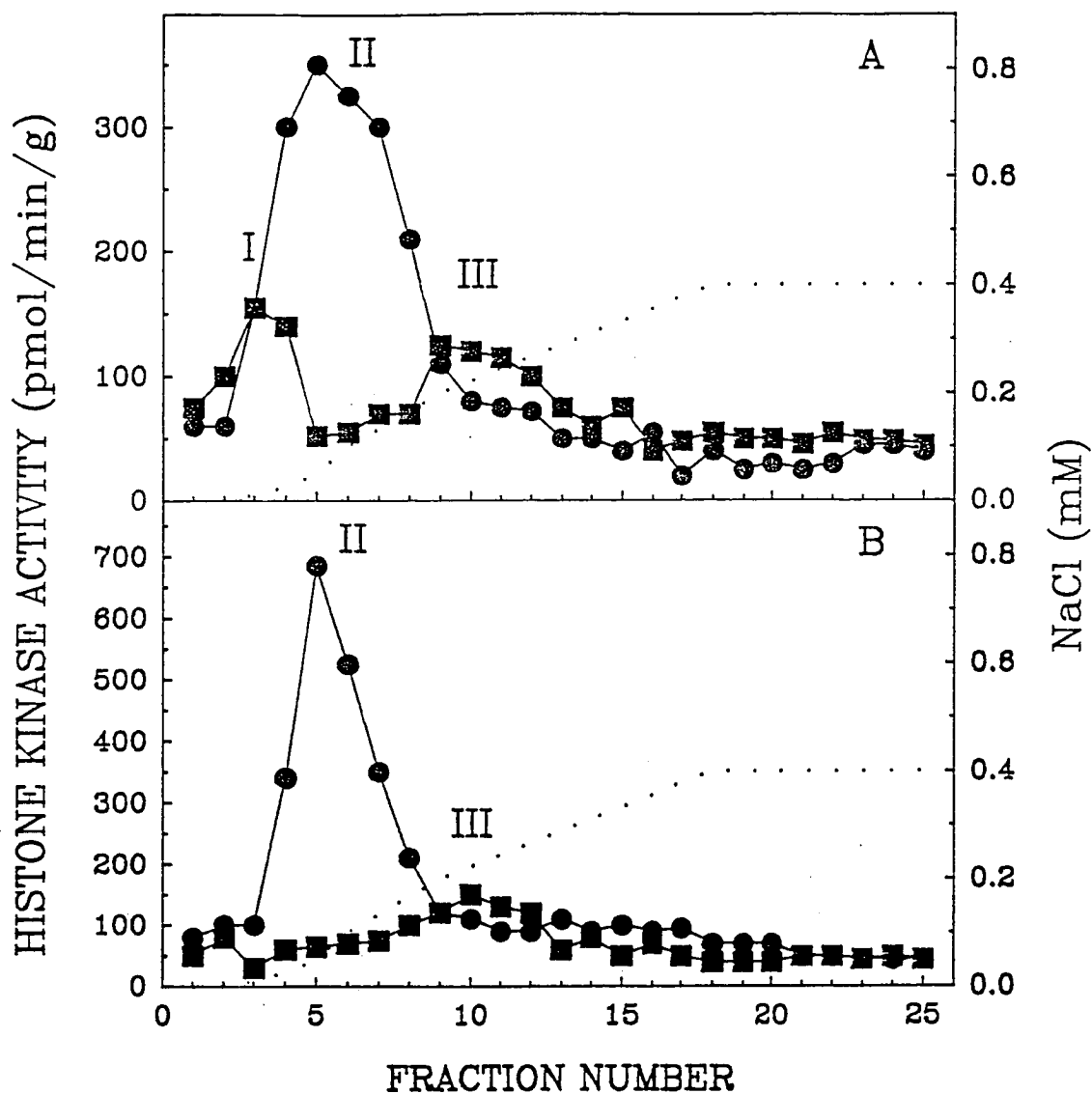


FIG. 2.2. DE-52-CELLULOSE CHROMATOGRAPHY OF HISTONE KINASE ACTIVITY FROM CYTOSOLIC (A) AND PARTICULATE (B) FRACTIONS OF RESTING GASTROCNEMIUS-PLANTARIS-SOLEUS SKELETAL MUSCLE GROUP. Enzyme activity was measured for Ca^{2+} -, phospholipid-dependent (●) and Ca^{2+} -, phospholipid-independent (■) histone kinase activity.

PK-A (Davison, 1987 Hons Thesis).

The histone kinase represented by peak III was found in both the cytosolic and the particulate fraction. The Ca^{2+} -phospholipid independence as well as the elution position (approx. 200 mM) of the enzyme was in agreement with previously published DE-52 chromatography characteristics for PKm from rat brain (Takai *et al.*, 1977), the catalytic active subunit of PKc. However, to further confirm the identity of peak III, limited tryptic proteolysis of PKc was performed to generate PKm. The application of the digested PKc to the same DE-52 chromatography procedures revealed new histone kinase peak that was Ca^{2+} -, phospholipid independent and which eluted in the range of 200-300 mM NaCl (Fig. 2.3). Therefore on the basis of the elution position from the DE-52 column and the ligand characteristics this kinase was identified as PKm (Takai *et al.*, 1977).

2.4.2 LIGAND DEPENDENCY OF PKc

To further establish the identity of the enzyme (Fig. 2.2, Peak II) as PKc the ligand dependency of cytosolic as well as particulate activity was assessed (Table 2.2). PKc values differ from Table 2.1 because only the DE-52 fraction (4 ml) with the highest PKc activity was used in these assays. The properties of the two activities were similar, although the cytosolic activity appeared to be more dependent on diolein. Full activity for histone phosphorylation was dependent on the presence of diolein,

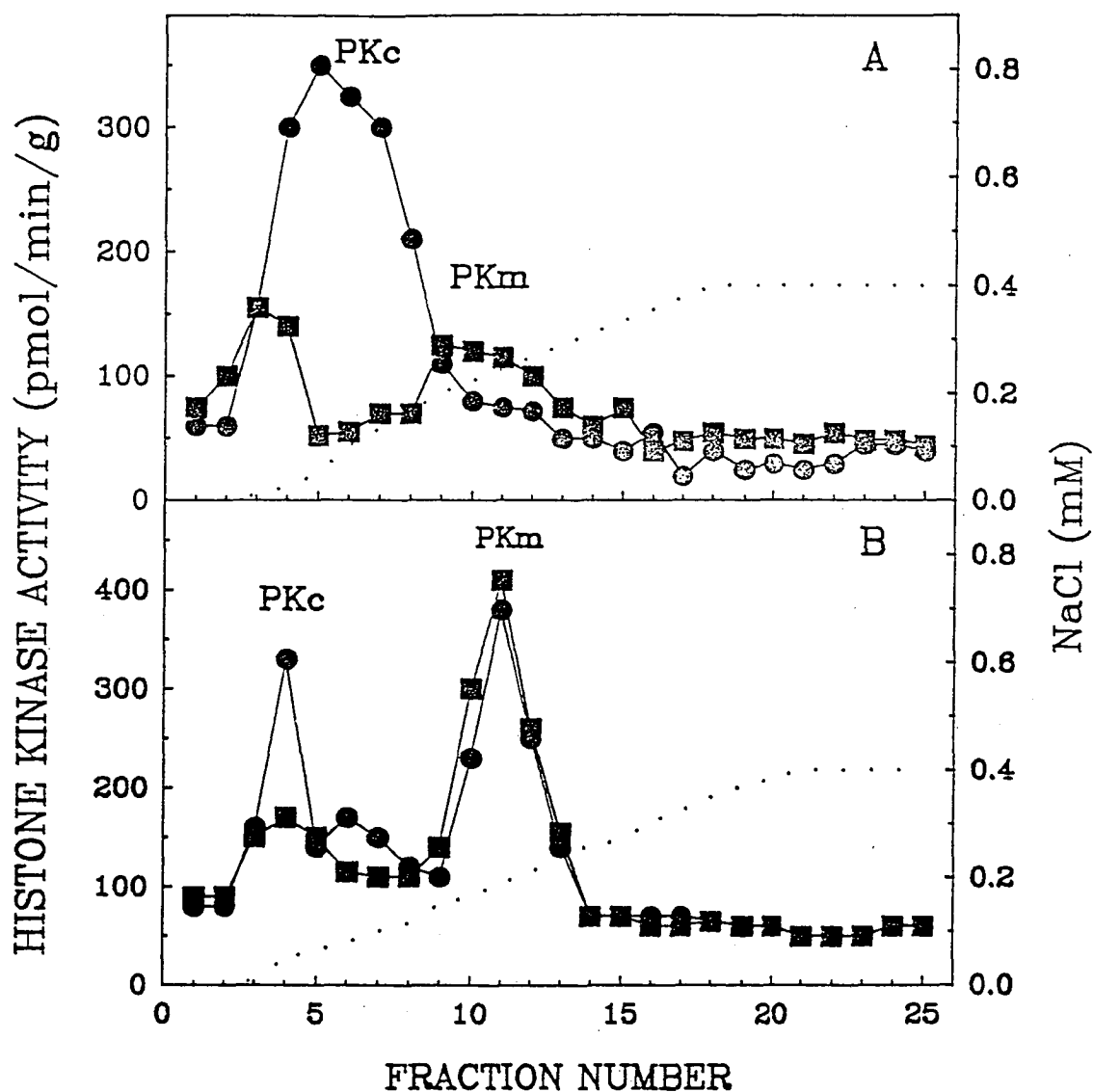


FIG. 2.3. GENERATION OF Ca^{2+} -, PHOSPHOLIPID-INDEPENDENT KINASE ACTIVITY (PK_m) BY PARTIAL TRYPTIC DIGESTION OF PARTIALLY PURIFIED CYTOSOLIC PK_c IN VITRO. Enzyme activity was measured for Ca^{2+} -, phospholipid-dependent (●) and Ca^{2+} -, phospholipid-independent (■) histone kinase activity.

TABLE 2.2 Ligand dependency of PKc from the cytosolic and particulate fractions of skeletal muscle

Conditions	PKc activity ^a (pmol/min per g muscle)	
	Cytosolic	Particulate
Complete assay	202 ± 9	530 ± 30
Minus – histone	13 ± 10	27 ± 4
Minus – phosphatidyl serine	15 ± 9	23 ± 7
Minus – diolein	30 ± 4	288 ± 10
Minus – phosphatidyl serine, –diolein	10 ± 3	23 ± 3
Minus – Ca ²⁺ , + 7.3 mM EGTA	8 ± 1	68 ± 9
Minus – Ca ²⁺ , –phosphatidyl serine, –diolein, + 7.3 mM EGTA	5 ± 6	38 ± 6
Minus – Ca ²⁺ , –phosphatidyl serine, –diolein, –histone, + 7.3 mM EGTA	1 ± 1	1 ± 1

Partially purified PKc was assayed as previously described (section 3.3) with changes to the complete assay as indicated in the Table.

^a Activity was determined on fractions from DE-52 chromatography. Values shown are means ± S.E. for three preparations.

phosphatidylserine and Ca^{2+} . Omission of these substances as well as histone completely inhibited protein phosphorylation. However when histone alone was omitted, endogenous protein phosphorylation by endogenous kinase(s) was evident but small compared with that measured in the presence of histone.

2.4.3 PKc CONTENT AND DISTRIBUTION IN THE GASTROCNEMIUS-PLANTARIS-SOLEUS MUSCLE GROUP AND ITS DIFFERENT MUSCLE TYPES

In Table 2.3 the content of PKc of the four different rat muscles that are part of the gastrocnemius-plantaris-soleus muscle group are shown, as well as the distribution of activity between cytosolic and particulate components. The order of activity from greatest to least was soleus > plantaris > gastrocnemius red = gastrocnemius white. The soleus muscle also had the greatest proportion of activity in the particulate fraction (67%) with plantaris, gastrocnemius red and gastrocnemius white each having approximately 52%. Since the gastrocnemius-plantaris-soleus muscle group comprises 5% soleus, 12.5% plantaris, 43.7% gastrocnemius white and 38.7% gastrocnemius red muscle (by weight) the combined data for the muscle group was 2806 pmol/min/g with approximately 53% of the total PKc activity in the particulate fraction. This compares favourably with the total activity for the gastrocnemius-plantaris-soleus muscle group determined as a whole (3250 ± 450 pmol/min/g, Table 2.1).

TABLE 2.3 PKc activity in muscles of the rat hindlimb

Muscle	Pkc activity		
	Cytosolic fraction	Particulate fraction	Total
	pmol/min/g		
Soleus	1909 ± 376	4015 ± 622	5956 ± 995
Plantaris	1854 ± 404	1999 ± 384	3851 ± 732
Gastrocnemius white	1121 ± 111	1249 ± 170 ^a	2392 ± 248 ^a
Gastrocnemius red	1236 ± 177	1304 ± 178 ^a	2538 ± 322 ^a

^a Significantly different from soleus ($P < 0.05$).

Each value represents the mean ± S.E. of determinations done in duplicate on muscles from six animals.

2.5 DISCUSSION

Minor modifications to existing methods have enabled quantitative recovery of cytosolic and particulate activities of PKc from skeletal muscle. The PKc activity noted in the present study was considerably greater than the published value of 373 pmol/min/g muscle determined on whole homogenate (Kuo *et al.*, 1980). To establish the identity of the enzyme as PKc, anion exchange chromatography using DEAE-cellulose (Whatman DE-52) was performed and indicated that both the cytosolic and solubilized particulate activity eluted at 40-50mM NaCl. In addition, the ligand dependency assessment showed that the enzyme appears to be characteristic of PKc (Inoue *et al.*, 1977; Takai *et al.*, 1979).

Detection of PKc isozymes in skeletal muscle has been limited to isozymes I, II and III. One report which used monospecific antibodies against each of these PKc isozymes found only PKc isozyme type II in skeletal muscle samples (Yoshida *et al.*, 1988).

PKm may represent one of the biologically active forms of PKc (Murray *et al.*, 1987). Therefore it was of particular relevance to this study. Its identification was aided by comparing the position of elution from the anion exchange column with the PKm generated *in vitro* by partial proteolysis of skeletal muscle PKc. As expected, PKm exhibited kinase activity independent of Ca^{2+} , phospholipid and DAG and present within both the cytosolic and particulate fractions.

Complete extraction of particulate PKc activity required relatively high concentrations of Triton X-100 (2%) and EGTA (10mM). The unstimulated gastrocnemius-plantaris-soleus muscle group had approximately 60% of its total PKc activity located in the particulate fraction. A high proportion of the total activity present in the particulate component implies, depending on the DAG, Ca^{2+} and phospholipid levels, a high state of activation of this enzyme even under resting conditions. Otherwise the enzyme may reside in an inactive state. The particulate component was assumed to be principally located in the muscle sarcolemma. This proved to be the case as a 50-fold purified sarcolemma vesicle preparation from skeletal muscle (Saito *et al.*, 1984; Savart *et al.*, 1985; Grimditch *et al.*, 1985) was found to contain high PKc activity (Rattigan, 1989).

Comparison of the activities of PKc in soleus and gastrocnemius white muscle indicates that the former has both a greater total and a greater particulate activity. Approximately 73% of the total activity was located in the soleus particulate fraction compared with 58% for the gastrocnemius white muscle. A greater total content and a greater apparent content of active (particulate) PKc may relate to the metabolic and physiological differences between these two muscle types.

There is speculation that transient intracellular Ca^{2+} elevation that accompanies muscle contraction may involve IP_3 (Vergara *et al.*, 1985; Volpe *et al.*, 1985; Nosek *et al.*, 1986; Thieleczek and Heilmeyer, 1986). However, the IP_3 theory is not without controversy as some authors are unable to find evidence to substantiate the involvement of IP_3 with Ca^{2+}

release (Volpe *et al.*, 1987; Mikos and Snow, 1987; Somlyo *et al.*, 1988). If IP_3 is produced upon muscle contraction then the second product from PI4,5-bisP hydrolysis is DAG. Moreover, the production of DAG would occur concomitantly with a rise in intracellular Ca^{2+} . Ca^{2+} and DAG are both regulators of PKc activity. Therefore, it is possible that muscle contraction may lead to PKc activation.

The next chapter examines the possibility that muscle contraction and the resultant mobilisation of intracellular Ca^{2+} are associated with DAG formation and subsequent PKc activation.

CHAPTER 3

EXERCISED-INDUCED TRANSLOCATION OF PKc AND PRODUCTION OF DIACYLGLYCEROL AND PHOSPHATIDIC ACID IN RAT SKELETAL MUSCLE IN VIVO

3.1 INTRODUCTION

Under physiological conditions PKc is activated by DAG generated from the hydrolysis of inositol phospholipids (Nishizuka, 1986). PKc activation is reflected by its translocation to the plasma membrane which is considered to be enriched in the particulate fraction (Nishizuka, 1984). The actual mechanism of enzyme translocation is unknown, however it is proposed to be a Ca^{2+} -mediated event. A model described by May *et al.* (1985) proposed that the increased intracellular Ca^{2+} recruits PKc to the plasma membrane, thus priming the system for activation by DAG.

Muscle contraction is associated with changes in the intracellular distribution of Ca^{2+} . Activation of skeletal muscle contraction under physiological conditions involves depolarization of transverse tubular regions of the sarcolemma (Huxley and Taylor, 1958; Hodgkin and Horowicz, 1960; Costantin and Taylor, 1973; Costantin, 1975) leading to Ca^{2+} release from the terminal cisternae of the sarcoplasmic reticulum (Stephenson, 1981).

In the present study we have examined the effect of contraction on the activation of PKc reflected by the translocation of this enzyme from the cytosol to the particulate fraction as well as the production of diacylglycerol and phosphatidic acid.

3.2 MATERIALS

L- α -Phosphatidyl-L-serine, L- α -phosphatidylinositol, L- α -phosphatidyl-ethanolamine, L- α -phosphatidylcholine, tripalmitin, diolein, 1,2-dioleoyl-sn-glycerol, dipalmitoyl phosphatidate, histone III-s, EGTA, dipalmitoyl phosphatidate, 1-O-hexadecyl-2-acetyl-sn-glycerol-3-phosphoryl choline and phospholipase C (type XII) were obtained from Sigma. [γ - 32 P]ATP was purchased from Bresatec (Adelaide, Australia). Silica Gel 60 F254 thin layer chromatography plates were from Merck; preswollen DE-52 cellulose and P81 phosphocellulose paper were obtained from Whatman.

3.3 METHODS

3.3.1 PREPARATION AND STIMULATION OF THE RAT GASTROCNEMIUS - PLANTARIS - SOLEUS SKELETAL MUSCLE GROUP IN VIVO

Fed, male hooded Wistar rats (250 ± 10 g) were anaesthetized with pentobarbital intra-peritoneally (12.5 mg). The skin was removed

peritoneally (12.5 mg). The skin was removed from the left hindlimb and the sciatic nerve exposed and cut to allow positioning of the distal end in a suction electrode. The gastrocnemius-plantaris-soleus muscle group was exposed by careful removal of overlaying tissue. The exposed muscle was kept moist with saline. The knee was secured by the tibiopatellar ligament and the Achilles tendon was attached via a steel wire to a Harvard Bioscience Isometric Transducer (Fig. 3.1). Tension development was recorded during electrical stimulation (Hugo Sachs Elektronik Nerve Muscle Stimulator Type 214). Unless indicated otherwise, the gastrocnemius-plantaris-soleus muscle group was stimulated by applying 200 ms trains of impulses (each impulse 0.1 ms duration) at a frequency of 100 Hz every 2 s. The voltage was adjusted (10-20 V) to attain full fibre recruitment. Although the initial tension was set at 500 g, this, as well as frequency of impulse and the number of trains, could be varied. If developed tension during full fibre recruitment decreased to less than 70% of the initial value the experiment was abandoned.

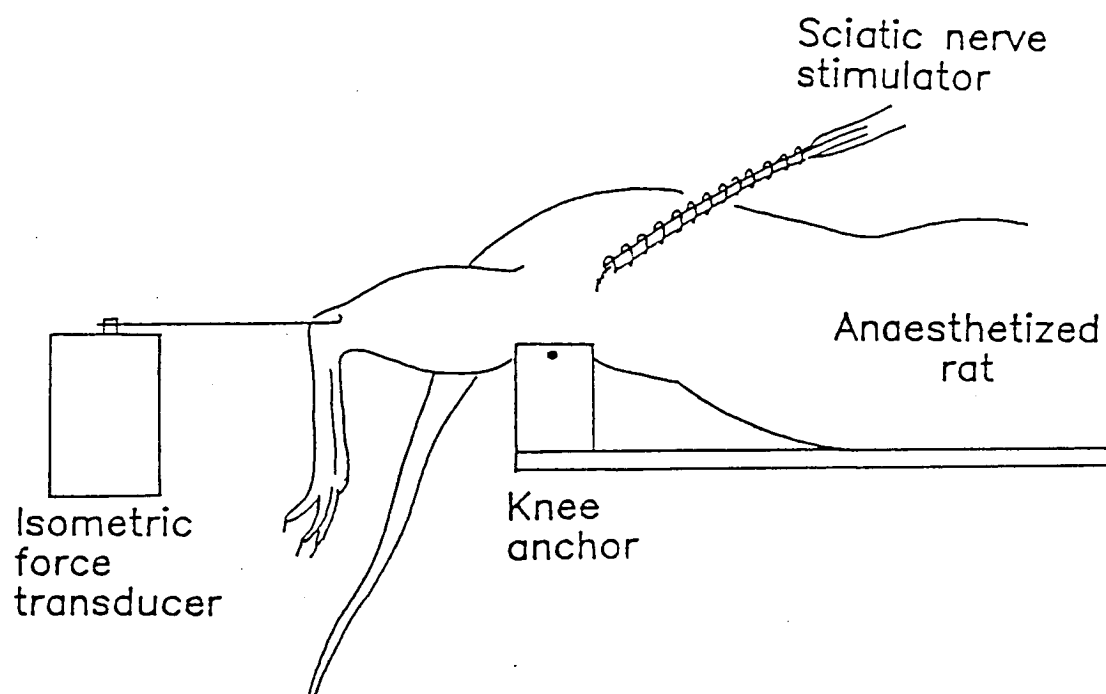


FIG. 3.1. DIAGRAMMATICAL REPRESENTATION OF THE RAT SKELETAL MUSCLE PREPARATION

3.3.2 EXTRACTION AND PARTIAL PURIFICATION OF PKc

The gastrocnemius-plantaris-soleus muscle group from non-exercised and exercised rats was rapidly removed (approx. 5 s) and processed as previously described in Sections 2.3.1 and 2.3.2).

3.3.3 DIACYLGLYCEROL AND PHOSPHATIDIC ACID DETERMINATION

Muscles required for diacylglycerol and phosphatidic acid analysis were initially freeze-clamped *in situ* using N₂-cooled tongs. Powdered frozen tissue (0.5 g) was then extracted (Ultra-Turrax, 2 X 30 s) with 1.2 ml chloroform:methanol (1:2). Further additions of chloroform (0.4 ml) and H₂O (0.4 ml) were made with vigorous mixing (Bligh and Dyer, 1959). Samples were then centrifuged (5 min, 2000 X g). The chloroform layer was removed and the aqueous layer re-extracted five times, as above. The chloroform extracts were filtered through glass wool and the solvent evaporated under N₂. The residue was dissolved in 0.5 ml chloroform and an aliquot (20 μ l) applied to a Silica Gel 60 F254 TLC plate. The solvent system for the resolution of diacylglycerol was toluene:diethyl ether:ammonium hydroxide (50:40:2:0.2). Plates were stained with Coomassie Blue R-250 (Nakamura and Handa, 1984) and the amount of diacylglycerol (1,2-isomer, R_f 0.67) was measured by laser densitometry (Figs 3.2 and 3.3). Reference standards were diolein (comprising approximately 80% 1,3-



FIG. 3.2. DAG AND PHOSPHATIDIC ACID SEPARATION BY THIN LAYER CHROMATOGRAPHY. Lipids are stained with Coomassie Blue

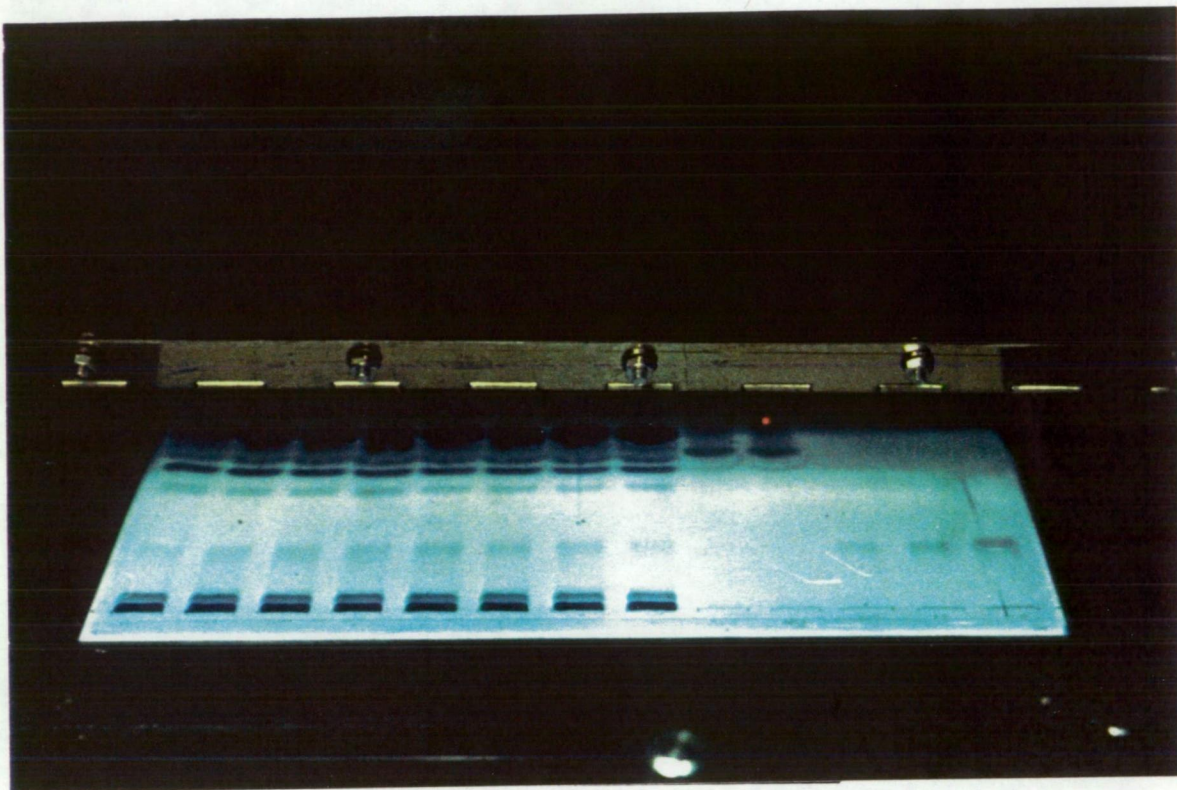


FIG. 3.3. QUANTITATIVE ANALYSIS OF COOMASSIE BLUE STAINED THIN LAYER CHROMATOGRAPHY PLATES BY LASER DENSITOMETRY

dioleoyl-glycerol (R_f 0.76) and approximately 20% 1,2-dioleoyl-glycerol (R_f 0.67)), 1,2-dioleoyl-sn-glycerol (R_f 0.67) and 1-O-hexadecyl-2-acetyl glycerol (R_f 0.88)). The intensity of the blue band corresponding to diacylglycerol was proportional to the mass applied to the plate for the range 0.125-1 μ g. Standards of 1,2-dioleoyl-sn-glycerol (0.125-1 μ g) were spotted on each plate and used to quantify the material of identical R_f present in tissue extract. Recovery of authentic 1,2-dioleoyl-sn-glycerol added to muscle homogenates was greater than 85% using the above procedure.

The solvent system for the resolution of phosphatidic acid was ethyl acetate:isooctane:acetic acid (45:25:10). Plates were stained with Coomassie Blue R-250 and the amount of phosphatidic acid (R_f 0.27) was measured by laser densitometry. The reference standard was 0.125-1 μ g of dipalmitoyl phosphatidate (R_f 0.27). Recovery of authentic material from muscle homogenates was also greater than 85% by this procedure. However, recovery of phospholipids by this method was variable and hence a different procedure was used.

3.3.4 PHOSPHOLIPID AND TRIACYLGLYCEROL EXTRACTION

Powdered frozen tissue (0.5 g) was extracted (Ultra-Turrax, 2 X 30 s; vortex 1-2 min) with 1.2 ml of chloroform, methanol, 35% HCl (200:100:1, v/v). Further additions of chloroform (0.4 ml) and water (0.4 ml) were made with vigorous mixing. Samples were then centrifuged (5

min, 2000 X g). The chloroform layer was removed and the aqueous layer reextracted five times, as above. The chloroform extracts were pooled and then evaporated to dryness under N₂. The residue was dissolved in 0.5 ml of chloroform and an aliquot (100 μ l) was applied to a Silica Gel 60 F254 TLC plate. Procedures for the resolution of phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine and for the separation of triacylglycerol were as described by Christie (1982).

3.3.5 FATTY ACID ANALYSIS

Diacylglycerol-, phosphatidic acid-, phospholipid-, and triacylglycerol-containing fractions were saponified (Christie, 1982) and the free fatty acids converted to their methyl esters (Morrison and Smith, 1964). Gas-liquid chromatography was performed using a 183 X 0.40 cm (inner diameter) column of 3% Silar 10C on Chromosorb G 80/100 mesh (Alltech Associates Inc.), a Hewlett-Packard model 5890 gas chromatograph, and flame ionisation detector. Fatty acid methyl esters were eluted at 180°C. Peak areas were integrated using a Skimadzu C-R3A Chromatopac Reporting Integrator.

3.4 RESULTS

3.4.1 PKc TRANSLOCATION

The effect of electrical stimulation-induced contraction on the translocation of PKc activity from the cytosol to the particulate fraction is illustrated in Fig. 3.4. Approximately 60% of the total PKc activity was located in the particulate fraction prior to contraction. This value increased in a time-dependent manner with contraction to reach a maximum of 86% at 10 min with a corresponding decrease in cytosolic activity. Although there was a tendency for the particulate activity to decrease after 10 min (Fig. 3.4A) while the cytosolic activity remained constant (Fig. 3.4B), the decrease was not significant. Thus, total activity remained the same throughout the 30 min contraction period and there was no evidence of PKm formation, as assessed by the appearance of a Ca^{2+} -, phospholipid-independent histone kinase eluting later from the DE-52 columns (Fig. 3.5).

Muscle contraction *in vivo* is accompanied by increased blood flow due to vasodilation and the blood content of the muscles increase. Thus, the weight of the gastrocnemius-plantaris-soleus muscle group was found to increase from 1.30 ± 0.05 (n=7) to 1.5, 1.6 and 1.82 ± 0.04 (n=6) at 1, 2 and 5 min respectively. To rule out the possibility that the increased blood content of the muscle group contributed to the observed translocation of PKc and the increases in diacylglycerol and phosphatidic acid, 0.35 g of blood from stimulated animals (cardiac puncture) was

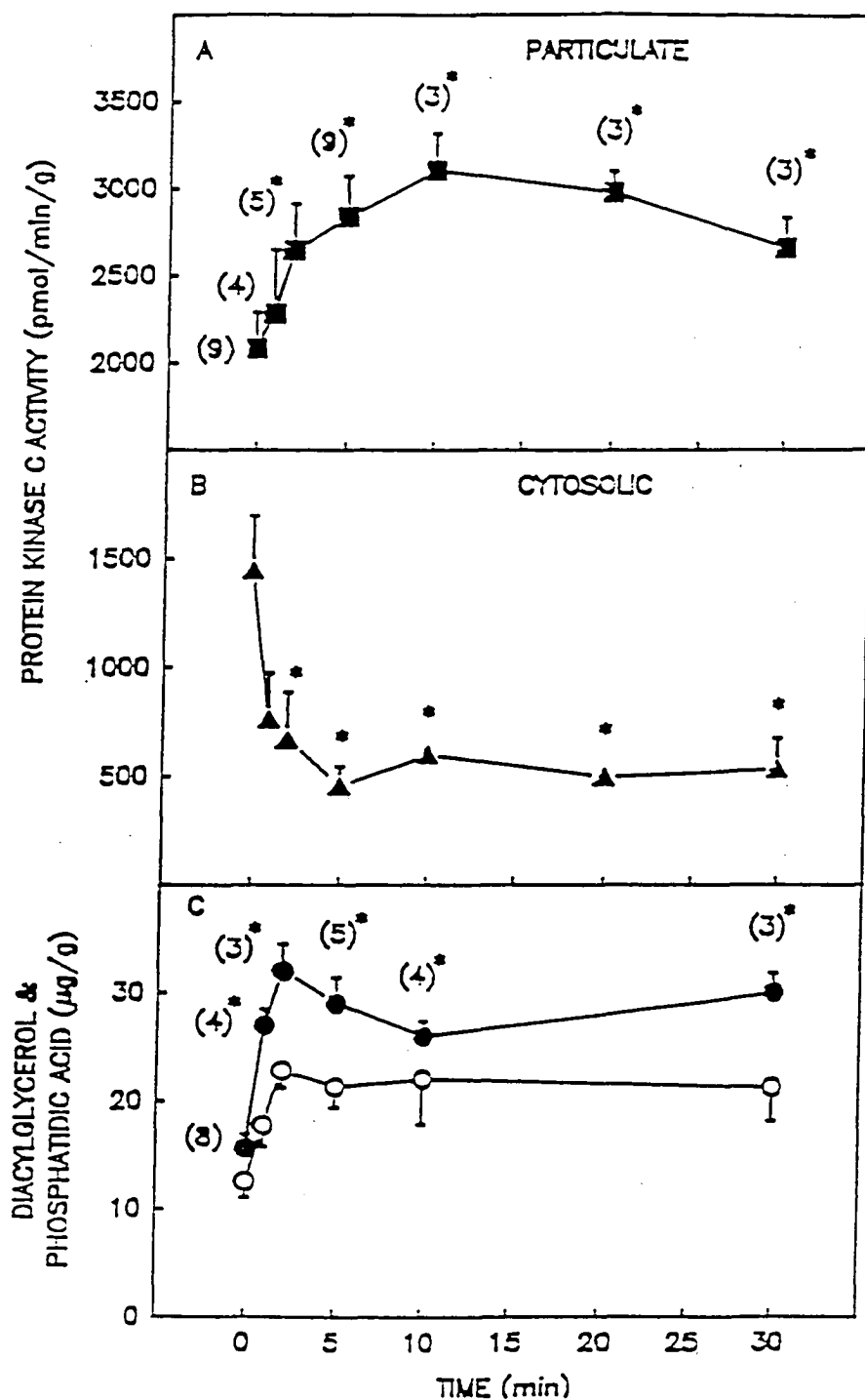


FIG. 3.4. TIME COURSE OF THE CONTRACTION-INDUCED TRANSLOCATION OF PKc AND FORMATION OF DAG (●) AND PHOSPHATIDIC ACID (○). Each value represents the mean \pm S.E.M. of determinations done in duplicate with the number of animals shown in parentheses. *, significantly different from zero time point ($P < 0.05$).

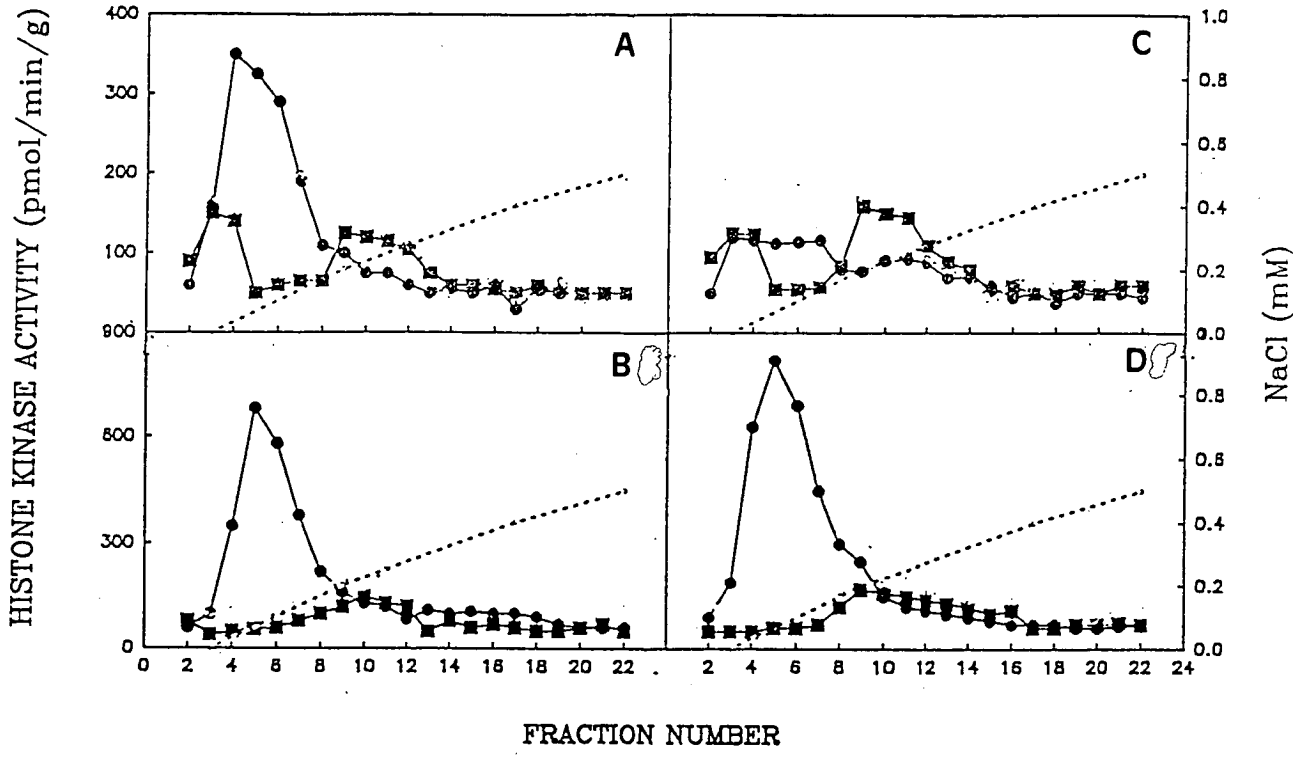


FIG. 3.5. ASSESSMENT OF PK_m FORMATION FOLLOWING 30 MIN MUSCLE CONTRACTION. DE-52 anion exchange profile of histone kinase activity from cytosolic (A,C) and particulate (B,D) preparations of resting (A,B) and contracting, 30 min (C,D) gastrocnemius-plantaris-soleus skeletal muscle. Enzyme activity was measured (see Section 2.3.7) for Ca^{2+} -, phospholipid-dependent (\bullet) and Ca^{2+} -, phospholipid-independent histone kinase activity (\blacksquare).

added to non-contracted muscle during homogenisation. This had no effect on any of the above parameters.

3.4.2 DIACYLGLYCEROL AND PHOSPHATIDIC ACID PRODUCTION

The effect of muscle stimulation on the production of diacylglycerol and phosphatidic acid is shown in Fig. 3.4C. Both substances showed a 2-fold increase in concentration over the first 2 min of electrical stimulation and maximal levels were then maintained for the remaining 28 min. The apparent trend for diacylglycerol concentration to decrease after 2 min was not statistically significant.

3.4.3 FATTY ACID COMPOSITION

The fatty acid composition of diacylglycerol and phosphatidic acid from resting and contracting (2 and 30 min) muscles is shown in Table 3.1 as well as the fatty acid composition of triacylglycerol, phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine. The diacylglycerol was rich in palmitic acid and contained stearic and arachidonic acids but did not contain either oleic or linoleic acids. In addition, the composition did not differ between resting and contracting muscles (2 and 30 min). The fatty acid composition of phosphatidic acid from resting muscle showed some resemblance to that of triacylglycerol;

TABLE 3.1 Fatty acid composition of phospholipids, diacylglycerol and phosphatidic acid

Fatty Acid	Phospholipids from resting Muscle				Diacylglycerol			Phosphatidic Acid		
	PI	PC	PE	TG	Non-Ex	Ex _{2 min}	Ex _{30 min}	Non-Ex	Ex _{2 min}	Ex _{30 min}
16:0	31.2	28.3	33.0	46.6	49.3	49.7	48.5	27.5	39.6	41.5
18:0	38.3	9.6	15.6	36.1	21.3	19.4	18.5	38.9	17.8	26.1
18:1	—	45.3	27.8	12.6	—	—	—	—	—	8.8
18:2	—	7.9	14.7	4.5	—	—	—	26.7	4.8	6.0
20:4	30.4	12.7	17.2	0.4	21.6	25.4	23.0	6.8	37.8	24.4

Muscles were from resting (Non-Ex) or electrically stimulated (Ex) gastrocnemius-plantaris-soleus groups *in vivo* and were stimulated for the times shown. Phospholipids, diacylglycerol and phosphatidic acid were fractionated by thin layer chromatography and collected. Transmethylation and gas-liquid chromatography were as described under "Experimental Procedures". Values are expressed as mol per 100 mol of the parent lipid and are the means of three determinations. Abbreviations: PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TG, triacylglycerol.

however, it did not contain oleate and instead contained an appreciable proportion of linoleic acid. As a result of exercise, differences between triacylglycerol and phosphatidic acid became more evident. For phosphatidic acid, there was a marked increase in the proportions of arachidonate and palmitate with decreases in stearate and linoleate. Thus, a comparison of the fatty acid composition of triacylglycerol with diacylglycerol at 2 and 30 min postexercise and with phosphatidic acid at 2 min postexercise would suggest that triacylglycerol is not the precursor. However, some formation of phosphatidic acid from triacylglycerol may account for the changes in fatty acid composition which are evident at 30 min postexercise.

3.4.4 EFFECT OF A PERIOD OF REST

Fig. 3.6 shows the results of experiments intended to examine the effect of a period of rest on muscles following electrical stimulation. Muscles were stimulated for 5 min with a total of 150 trains of tetanic stimuli and then allowed periods of 10, 20 and 30 min of rest before excision. Electrical stimulation increased the particulate fraction content of PKc (Fig. 3.6A) and produced a corresponding decrease in the cytosolic fraction content of the enzyme (data not shown). There was also a 2-fold increase in the muscle concentration of DAG and phosphatidic acid (Fig. 3.6B). However, resting the muscle for periods up to 30 min after stimulation did not lead to a reversal of any of these

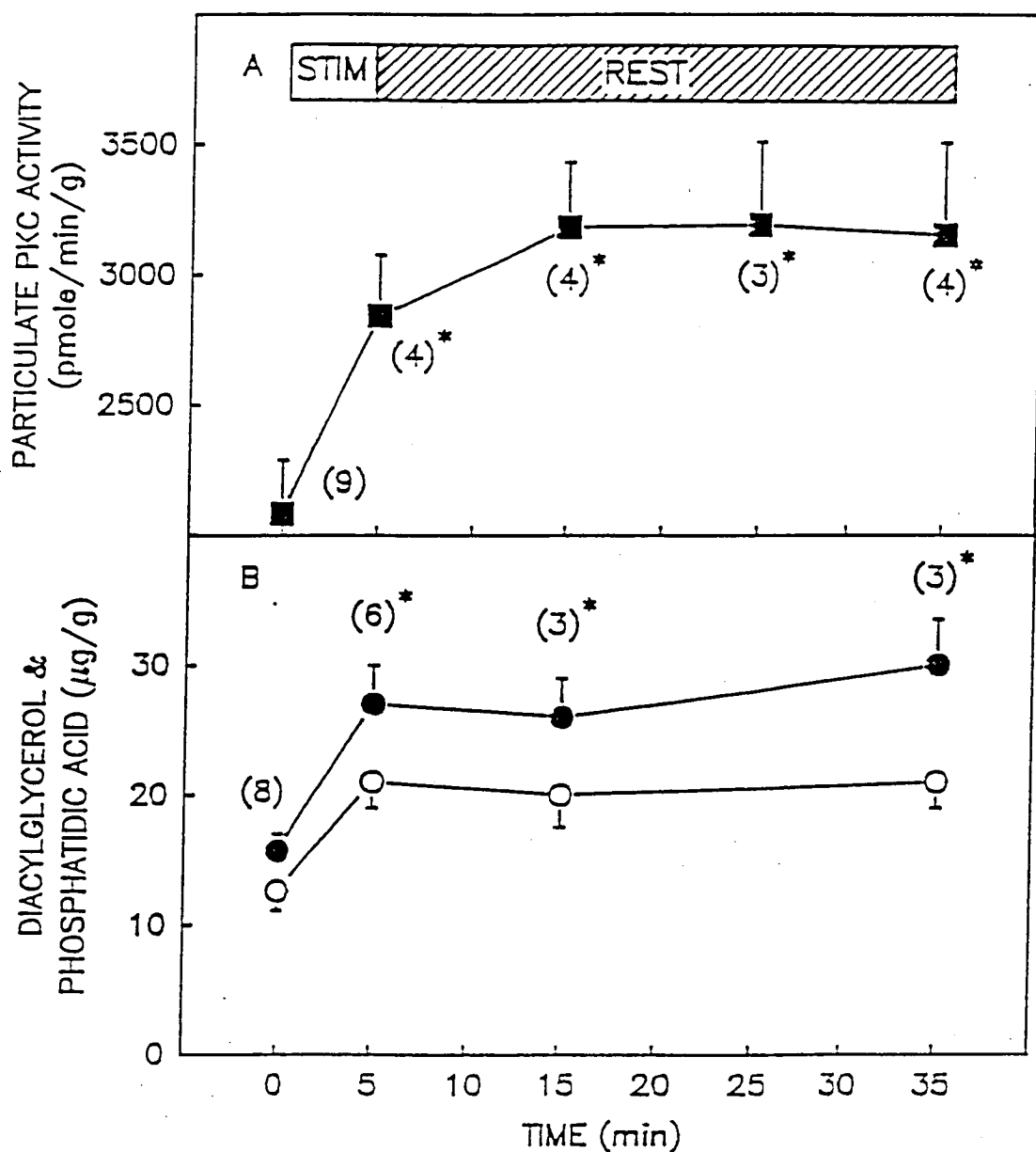


FIG. 3.6. EFFECT OF STIMULATION FOLLOWED BY A PERIOD OF REST ON THE TRANSLOCATION OF PKc AND FORMATION OF DAG (●) AND PHOSPHATIDIC ACID (○). Each value represents the mean \pm S.E.M. of determinations done in duplicate with the number of animals shown in parentheses. *, significantly different from zero time point ($P < 0.05$). There was no significant difference in the total activity recovered at each time (3642 ± 200 pmol/min/g, mean \pm S.E.M., $n = 24$).

changes.

3.4.5 EFFECT OF APPLIED LOAD

Three parameters that influence muscle response to electrical stimulation are applied load, frequency of non-tetanic stimuli and the total number of tetanic stimuli. Accordingly, each of these has been varied independently and the effects examined.

Table 3.2 shows the effect of varying the initial load at maximum fibre recruitment. For these experiments, the isometric force transducer was standardised against weights of 100, 500 and 1000 g and then positioned so as to apply an initial tension on the gastrocnemius-plantaris-soleus muscle group of 0, 200, 500 or 700 g. The sciatic nerve was then stimulated for a 5 min period with a total of 150 tetanic trains. The data show that translocation of PKc from the cytosolic to the particulate fraction was not altered by the initial load.

3.4.6 EFFECT OF THE FREQUENCY OF NON-TETANIC STIMULI

Fig. 3.7 shows the effect of the frequency of non-tetanic stimuli on the translocation of PKc activity from the cytosol to the particulate fraction. In all other experiments reported in this study (Figs 3.4 and 3.6), the frequency of impulses (each 0.1 ms) was 100 Hz. A frequency of this

TABLE 3.2 Effect of load applied to the muscle during electrical stimulation

	Load applied to muscle	PKc activity		
		Cytosolic fraction	Particulate fraction	Total
	g		pmol/min/g	
Non-stimulated	500	1450 ± 250	2070 ± 200	3250 ± 450 (9)
Stimulated	0	328 ± 100	2941 ± 260 ^a	3269 ± 360 (3)
“	200	362 ± 76	3110 ± 65 ^a	3472 ± 141 (3)
“	500	455 ± 90	2843 ± 233 ^a	3293 ± 257 (9)
“	700	361 ± 112	3122 ± 194 ^a	3483 ± 306 (3)

Values given are means ± S.E. with the number of animals shown in parentheses.

^a All stimulated values were significantly greater than non-stimulated values ($P < 0.01$) but were not significantly different from each other.

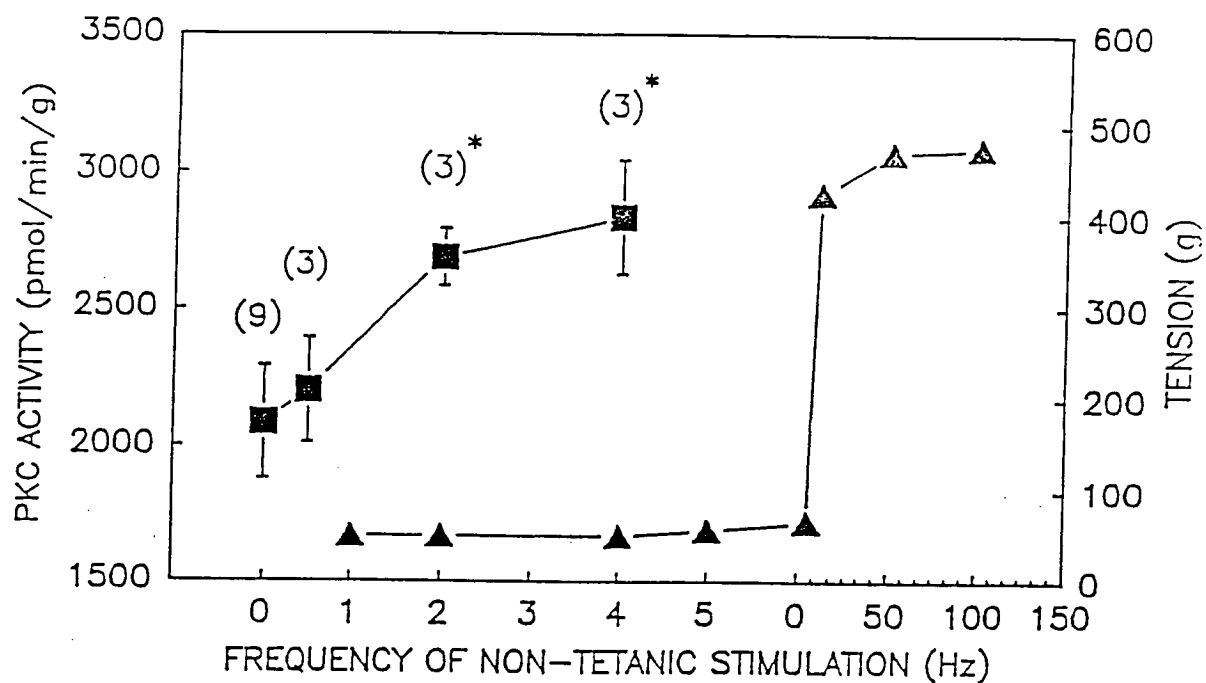


FIG. 3.7. EFFECT OF FREQUENCY OF NON-TETANIC STIMULI ON TENSION DEVELOPMENT (\blacktriangle) AND ON THE TRANSLOCATION OF PKC (\blacksquare). Each value represents the mean \pm S.E.M. of determinations done in duplicate with the number of animals shown in parentheses. *, significantly different from unstimulated muscles ($P < 0.05$). There was no significant difference in the total activity recovered at each stimulation (3358 ± 215 pmol/min/g, mean \pm S.E.M., $n = 18$).

magnitude is regarded as tetanic. Fig. 3.7 shows that the threshold for tetanic stimuli is between 10 and 20 Hz and that maximal translocation of PKc occurred at a frequency that was subtetanic.

3.4.7 EFFECT OF THE NUMBER OF TETANIC STIMULI

The experiment in Fig. 3.8 examines the relationship between the number of tetanic stimuli and the translocation of PKc. It was found that only one tetanic stimulus of 200 ms duration at the start of the 30 min period was required to translocate the enzyme fully (Fig.3.8A). Thus, changes in diacylglycerol and phosphatidic acid were also assessed and shown in Fig. 3.8B. Again only one stimulus was required to give maximal increases in both substances.

3.5 DISCUSSION

These results demonstrate for the first time that electrical stimulation of the sciatic nerve leads to changes consistent with PKc activation in the gastrocnemius-plantaris-soleus muscle group of rat hindlimb. A principal finding was the time-dependent translocation of the enzyme from the cytosol to the particulate fraction accompanied by a 2-fold increase in the concentration of diacylglycerol and phosphatidic acid. The association between the change in concentration of

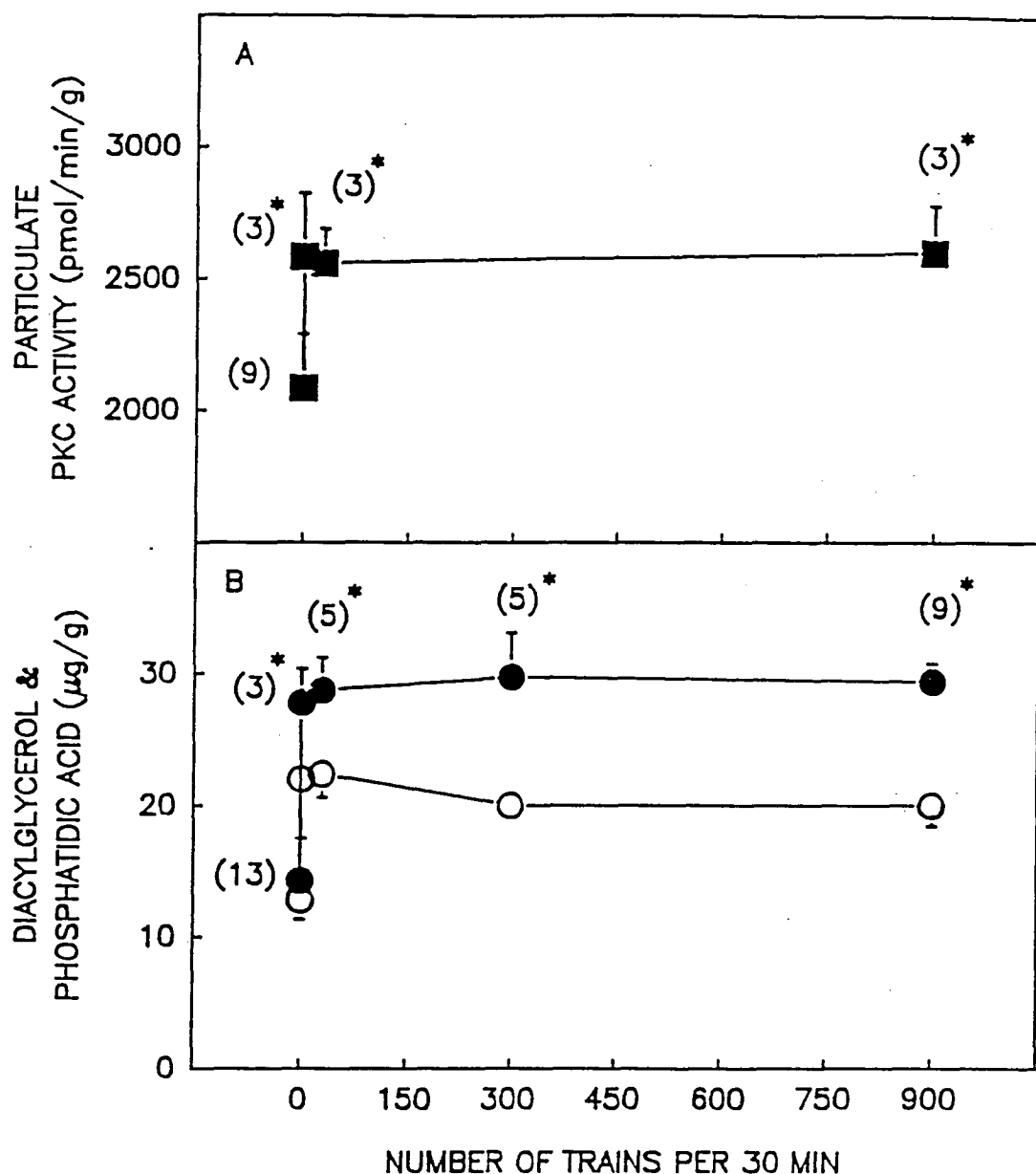


FIG. 3.8. EFFECT OF THE NUMBER OF TETANIC STIMULI ON THE TRANSLOCATION OF PKc (■) AND FORMATION OF DAG (●) AND PHOSPHATIDIC ACID (○). Each value represents the mean \pm S.E.M. of determinations done in duplicate with the number of animals shown in parentheses. *, significantly different from unstimulated muscles ($P < 0.05$). There were no significant differences in total activity recovered at each stimulation (3287 ± 205 pmol/min/g, mean \pm S.E.M., $n = 35$).

diacylglycerol and the translocation appeared to be causal as the increase in diacylglycerol (i) preceded the translocation of PKc; (ii) remained elevated when stimulation of the sciatic nerve halted and thus could be the cause of a sustained translocation of PKc; and (iii) occurred as the result of a single tetanic stimulus, the latter also being sufficient to translocate the enzyme fully.

Diacylglycerol is regarded as a key regulator of PKc (Nishizuka, 1984). Its production from membrane phospholipids together with Ca^{2+} are considered to be the trigger for PKc activation by initiating a translocation from the cytosol to the membrane in which other activators of the enzyme such as phosphatidylserine are located. Thus, providing Ca^{2+} is also present, a quarternary complex consisting of Ca^{2+} , phospholipid, DAG and the kinase itself (Niedel and Blackshear, 1986) leads to activation of PKc. In the present study, the origin of the increased concentration of DAG is not clear. The measurements of DAG present net accumulation and do not permit an assessment of the effects of muscle contraction on DAG formation and breakdown. The fatty acid analyses indicate that inositol phospholipids are probably precursors to the DAG pools in skeletal muscle because of the relatively high content of arachidonic acid. However, the high content of palmitate may suggest that triacylglycerol hydrolysis is also a contributor, although the total absence of oleate in the DAG weakens this argument. The origin of the phosphatidic acid is also unclear. Explanations must be consistent with observations that the increase in concentration of this substance closely parallels that of DAG (Fig. 3.4). The fatty acid

composition of the newly formed phosphatidic acid was similar to that of the DAG. These findings suggest that both substances were hydrolysis products of the same phospholipid. If this is the case, then DAG and phosphatidic acid may be formed simultaneously by the actions of phospholipases C and D, respectively, or phospholipase C and DAG kinase, respectively. Agonist-mediated rapid formation of phosphatidic acid has been reported in other systems (Bocckino *et al.*, 1987). There is also some evidence that phosphatidic acid can substitute for DAG in the activation of PKc (Sekiguchi *et al.*, 1988).

Perhaps an unexpected finding was that the concentrations of both DAG and phosphatidic acid remained elevated even after resting the muscle for periods up to 30 min poststimulation. This is unusual, as in other systems metabolism of DAG and phosphatidic acid appears to be rapid (Besterman *et al.*, 1986 and May *et al.*, 1986). Such an occurrence would imply that stimulation leads to a prolonged effect on the lipid metabolising enzymes responsible for DAG and phosphatidic acid production.

An important observation from the present study was that maximal translocation of PKc occurred at subtetanic frequencies (Fig. 3.7, page 69). Two possibilities could account for this phenomenon. Firstly, at subtetanic frequencies diacylglycerol has been produced in sufficient quantity to activate the translocation of PKc to the membrane without a significant change in intracellular Ca^{2+} concentration. This could imply that basal Ca^{2+} levels may be sufficient to allow the ternary complex involving PKc, DAG, phosphatidylserine and Ca^{2+} to form.

Secondly, subtetanic frequencies may simply increase Ca^{2+} but not to the level required to activate the troponin C complex. However, this change could be sufficient to promote the translocation and activation of PKc. Further studies will be required to compare the Ca^{2+} dependent translocation of PKc with that for troponin myosin contraction.

In the present study, we have shown that muscle contraction *in vivo* leads to translocation of PKc and the formation of its activator, DAG. Among the possible substrates of PKc is the glucose transporter protein (Witters *et al.*, 1985). Although phosphorylation of the glucose transporter itself does not seem to lead to changes in the transporter intrinsic activity (Joost *et al.*, 1987), there is overwhelming evidence to suggest that PKc is some how involved with glucose transport, particularly with regard to insulin (Klip and Douen, 1989).

The next chapter examines the relationship between muscle contraction, PKc activation and changes in the rate of muscle glucose transport *in vivo*.

CHAPTER 4

PKc ACTIVATION AND ITS RELATIONSHIP TO CHANGES IN GLUCOSE TRANSPORT

4.1 INTRODUCTION

While the data presented in the previous chapter illustrate, *in vivo* that electrically-induced skeletal muscle contraction can lead to PKc activation, it nevertheless remains to be seen whether PKc is involved in the regulatory mechanism for contraction-induced glucose uptake. Previous reports have demonstrated that the glucose transporter is a substrate for PKc (Witters *et al.*, 1985). However, there have been no reported attempts to correlate PKc activation and changes to glucose transport due to muscle contraction, *in vivo*.

Studies reported in this chapter are designed to determine if a correlation exists between the activation of PKc and contraction-induced glucose transport. Two systems were examined. The *in vivo* gastrocnemius-plantaris-soleus muscle group preparation and the *in vitro* isolated soleus muscle preparation.

4.2 MATERIALS

2-Deoxy[1-³H]glucose, [U-¹⁴C]sucrose and 3-O-methyl[³H]glucose were from the Amersham Corp.

4.3 METHODS

4.3.1 GLUCOSE TRANSPORT AND UPTAKE

Glucose uptake *in vivo* was determined using the method of Ferre' *et al.* (1985), derived from the technique of Sokoloff *et al.* (1977). The theoretical basis of the method is outlined by Ferre' *et al.* (1985) and the rate of glucose uptake (R_i) from the injection of label (time=0) until the sampling time (τ) in the present study was calculated using their steady-state equation.

$$R_i = \frac{[2\text{-deoxyglucose 6-phosphate}]_\tau}{LC \int_0^\tau (C_B^*/C_B) dt}$$

where LC (the lumped constant) is a correction for the discrimination against 2-deoxyglucose in glucose transport and phosphorylation pathways. In the present experiments, the lumped constant was set at 1.0, as the average of the values determined by Ferre' *et al.* (1985) for soleus (0.95) and extensor digitorum longus (1.05); C_B^* and C_B represent the blood 2-deoxyglucose expressed in terms of radioactivity and the blood glucose concentration, respectively.

4.3.2 IN VIVO DETERMINATION OF 2-DEOXYGLUCOSE UPTAKE

A bolus injection of 30 μCi of 2-deoxy[1- ^3H]glucose was administered via a carotid artery catheter into the anaesthetised animal. Samples of blood were then removed at intervals of 5 min to monitor the concentration of both 2-deoxyglucose and glucose (Fig. 4.1). Electrical stimulation of the sciatic nerve (see Section 3.3.1) immediately followed injection of the labelled 2-deoxyglucose. The gastrocnemius-plantaris-soleus muscle group was excised at steady state (30 min, Fig. 4.1) for the determination of 2-deoxyglucose 6-phosphate concentration.

4.3.3 DETERMINATION OF 2-DEOXYGLUCOSE AND 2-DEOXYGLUCOSE 6-PHOSPHATE

To frozen powdered heart (100 mg) was added 0.5 ml cold 6% PCA. Samples were homogenised and then neutralised by the addition of 5 M KOH ($90 \pm 5 \mu\text{l}$ aliquots), using methyl orange as indicator (0.1 % in ethanol, 5 μl). Upon centrifugation (2000 X g, 5 min) 0.4 ml aliquots of supernatant were applied to 1 ml Dowex AG1X8 (formate form) and Dowex AG50W-X1 (hydrogen form) ion-exchange columns (Ferre' *et al.*, 1985). 2-Deoxyglucose was eluted with 6 X 0.5 ml H_2O . 2-Deoxyglucose 6-phosphate was eluted with 9 X 0.5 ml of 0.5 M ammonium acetate, pH 4.9. Aliquots (1.5 ml) were added to 12 ml ASC II (Amersham Corp.) for counting.

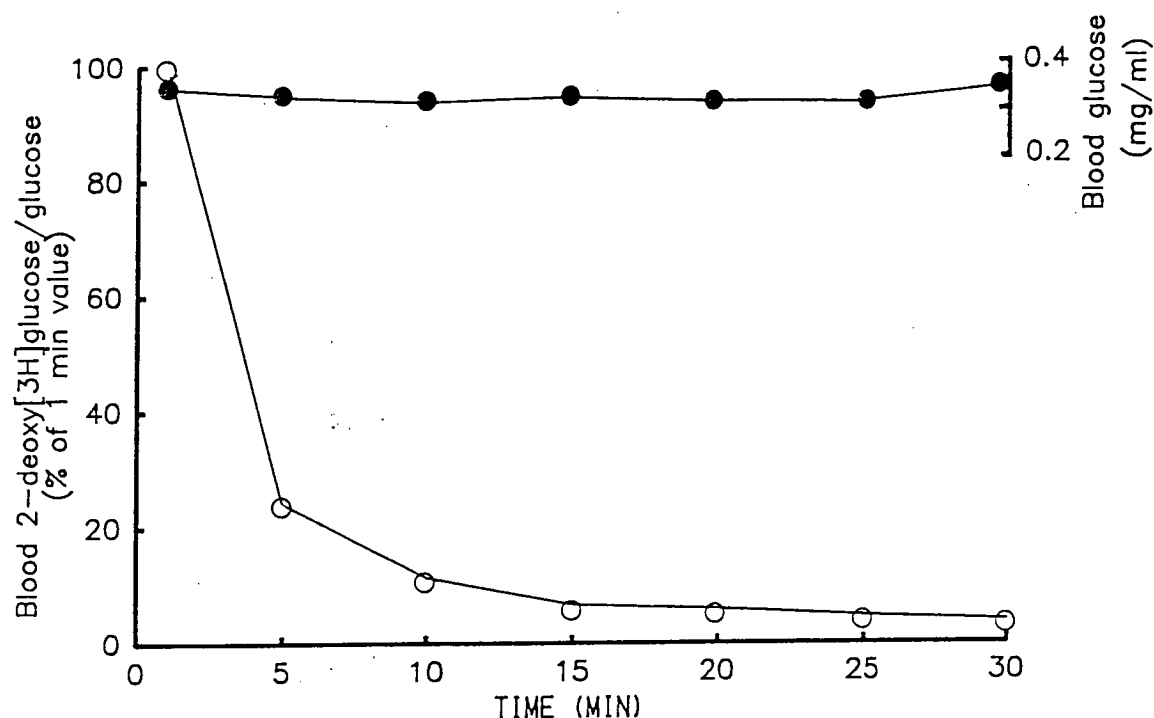


FIG. 4.1. TIME COURSE OF THE RATE OF DISAPPEARANCE OF BLOOD 2-DEOXY[^3H]GLUCOSE (○) AS WELL AS BLOOD GLUCOSE CONCENTRATION (●). A bolus injection of 2-deoxy[^3H]glucose was administered via a carotid artery catheter into the anaesthetised rat. At time intervals indicated, blood samples were taken to monitor the concentration of both 2-deoxy[^3H]glucose and glucose. The results are representative of several determinations.

4.3.4 IN VITRO DETERMINATION OF GLUCOSE UPTAKE

Glucose uptake *in vitro* was determined using isolated soleus muscle (approximately 40 mg) from juvenile male hooded Wistar rats (80-100 g body weight). Muscles were removed from the animal and quickly mounted by the tendons on a muscle suspension apparatus similar to that described by Nesher *et al.* (1980). The incubation medium (4 ml) was modified Krebs-Ringer bicarbonate buffer of the following composition: 93 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 0.05 mM Na_2EDTA , 1.27 mM CaCl_2 , 25 mM NaHCO_3 , 5 mM HEPES, 5 mM pyruvate, 50 mM 3-O-methyl[1- ^3H]glucose (0.4 $\mu\text{Ci/ml}$) and 5 mM [U- ^{14}C]sucrose (0.125 $\mu\text{Ci/ml}$). The medium was gassed with 95% O_2 , 5% CO_2 and the pH was adjusted to 7.4. Gas flow was broken into extremely small bubbles providing thorough mixing of the incubation medium. Two muscles were mounted simultaneously, and details for electrical stimulation were similar to those described above for studies *in vivo* except that impulses were of 1 ms duration (Fig. 4.2). [U- ^{14}C]Sucrose was added to enable the extracellular space in the muscle to be measured. The frozen muscles were homogenised in 2 ml of H_2O (Ultra-Turrax, 2 X 30 s), centrifuged (15 min, 220 X g), and 1.5 ml of the supernatant was added to 12 ml ASC II (Amersham Corp.) for counting.

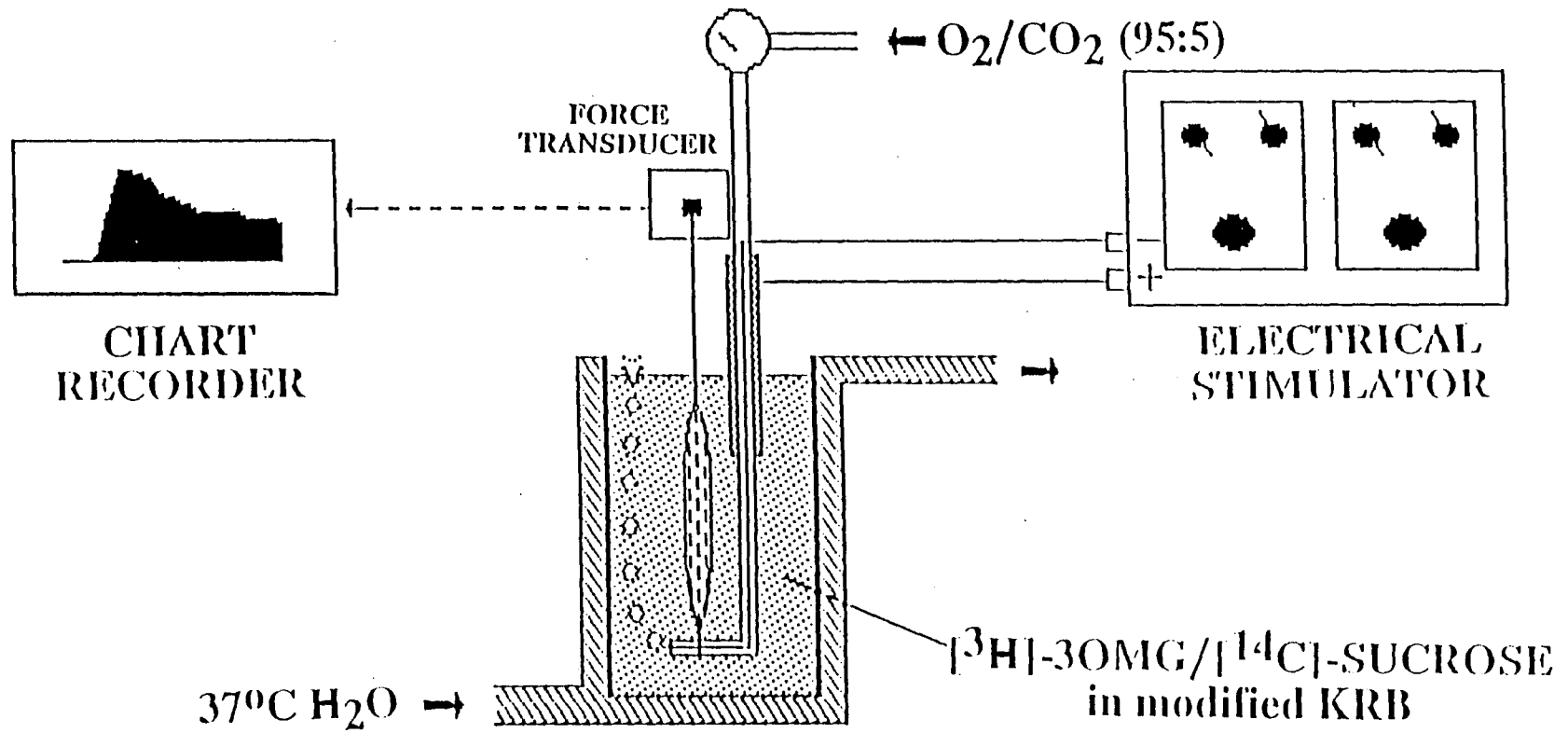


FIG. 4.2. MUSCLE SUSPENSION APPARATUS

4.4 RESULTS

4.4.1 GLUCOSE TRANSPORT IN THE GASTROCNEMIUS-PLANTARIS-SOLEUS MUSCLE GROUP IN VIVO

Glucose transport as determined by the accumulation of 2-deoxyglucose 6-phosphate in the gastrocnemius-plantaris-soleus muscle group *in vivo* was assessed as a function of the number of tetanic stimuli. Fig. 4.3 shows that a minimum of 35 stimuli was required to activate glucose transport and that the maximal rate of uptake was still not reached even at 900 stimuli/30 min.

4.4.2 GLUCOSE TRANSPORT IN THE ISOLATED SOLEUS MUSCLE

One of the disadvantages of the *in vivo* method for determining glucose uptake is that time courses cannot be conducted with precision. Thus, to determine the time course for electrical stimulation-activation of glucose transport, the isolated soleus muscle was used. Despite differences in experimental conditions between the *in vivo* and the soleus *in vitro* systems, it was apparent that the activation of glucose transport by electrical stimulation was relatively slow to develop and required at least 20 min to reach maximum values (Fig. 4.4).

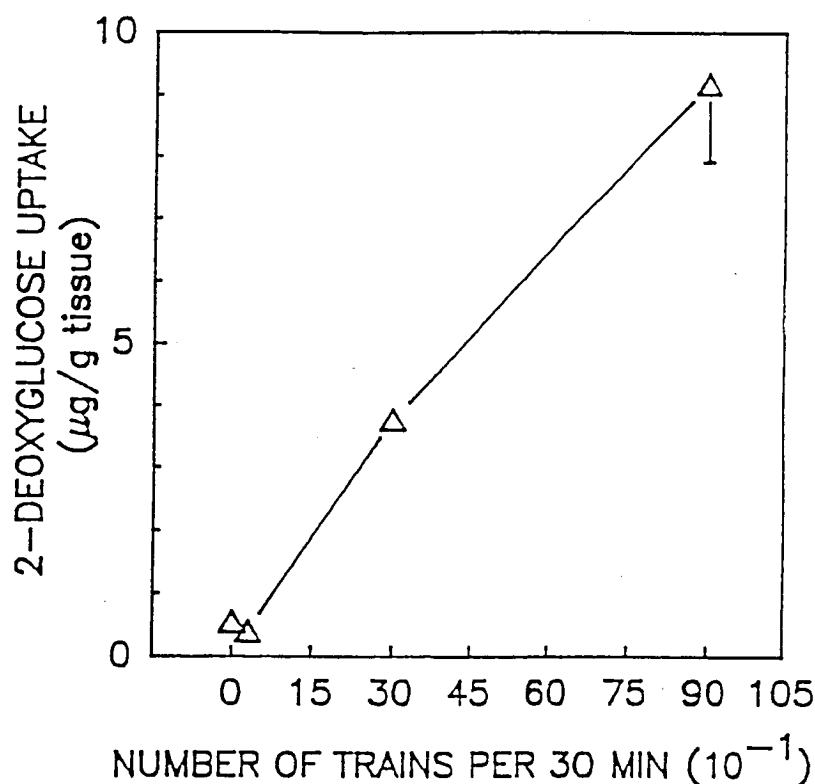


FIG. 4.3. EFFECT OF THE NUMBER OF TETANIC STIMULI ON THE UPTAKE OF 2-DEOXYGLUCOSE BY THE GASTROCNEMIUS-PLANTARIS-SOLEUS MUSCLE GROUP IN VIVO. Determination of 2-deoxyglucose uptake was as described in section 4.3.2. and 4.3.3. The muscle was stimulated as described in section 3.3.1. Electrical stimulation of the sciatic nerve commenced immediately following the injection of labelled 2-deoxyglucose. Each train of electrical stimuli (tetanic stimuli) of 200 ms duration comprised 20 impulses of 0.1 ms duration at 10-20 V. Multiple trains were spaced 2 sec apart. The muscle group was excised at steady state (30min) for the determination of 2-deoxyglucose 6-phosphate concentration. Values shown are means \pm S.E. for a minimum of three animals at each point.

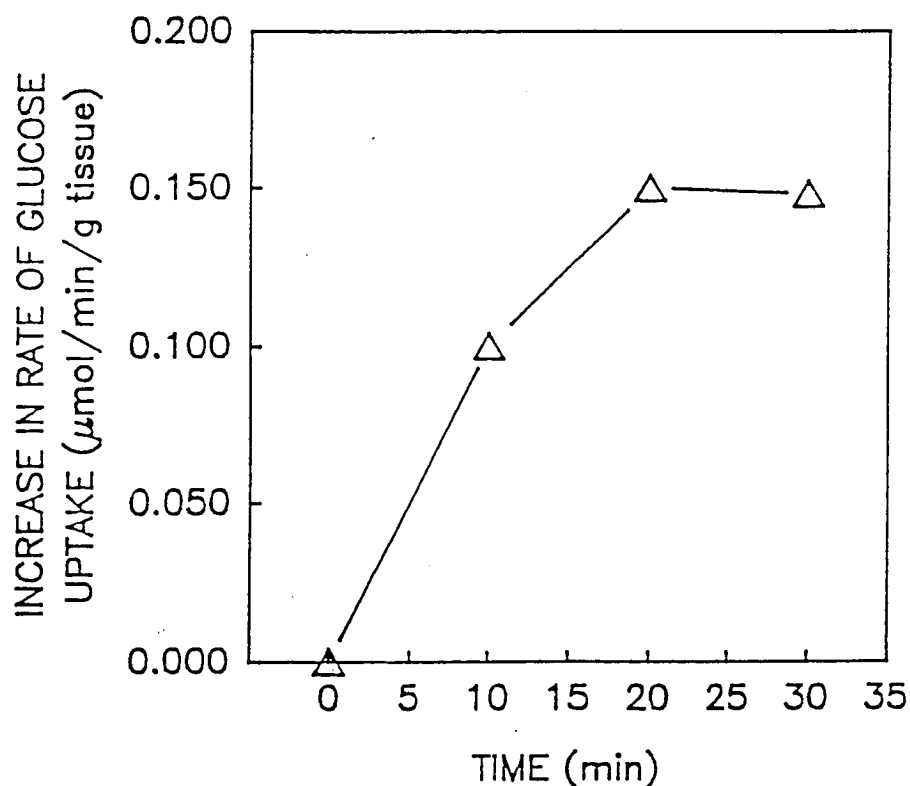


FIG. 4.4. TIME COURSE OF CONTRACTION-INDUCED INCREASE IN GLUCOSE TRANSPORT BY ISOLATED SOLEUS MUSCLES. Muscles were mounted by the tendons on a muscle suspension apparatus as described in section 4.3.4. Tension development was recorded during electrical stimulation (200 ms trains of impulses, each of 1 ms duration at a frequency of 100 Hz every 2 sec). The voltage was adjusted (10-20 V) to attain full fibre recruitment. Muscles were then removed, rinsed in fresh buffer, blotted dry and homogenized for determination of radioactivity. Results shown are mean \pm S.E. from six muscles at each time point (error bars are within the symbols) and are expressed in terms of the increase in the rate of uptake where the basal rate was $0.13 \pm 0.02 \mu\text{mol/min/g}$.

4.5 DISCUSSION

The present study examined the relationship among muscle contraction, PKc activation and changes in the rate of muscle glucose transport.

In Chapter 3, it was shown that the increase in DAG and phosphatidic acid preceded PKc translocation *in vivo*. Time-dependent changes in glucose transport rates could not be determined *in vivo*. However, time course studies of the activation of this process in the isolated soleus muscle suggested that it was slower to develop than PKc translocation. Thus, a causal relationship between electrical stimulation and the increase in glucose transport involving PKc activation may exist. Even so, the data of Figs 4.3 and 4.4 appear to be less supportive of that view as activation of PKc required far fewer tetanic stimuli than did glucose transport.

Whilst the present findings suggest that PKc translocation and activation are involved in the activation of glucose transport in skeletal muscle, it is important to note that the evidence presented herein could be circumstantial. PKc is found in high concentration in vascular smooth muscle and neural tissue (Kuo *et al.*, 1980). Thus, electrical stimulation may lead to the activation of PKc in tissue other than that in which glucose transport and contractility are occurring. If this is the case then it may explain the apparent dissociation between PKc translocation and contractility. Clearly, more studies are required to confirm the tissue location of PKc in rat hindlimb.

The next chapter examines, more closely, the role of PKc in contraction-induced glucose uptake by using a relatively direct TPA-PKc "down-regulation" method.

CHAPTER 5

LONG-TERM TREATMENT OF ISOLATED RAT SOLEUS MUSCLE WITH PHORBOL ESTER LEADS TO LOSS OF CONTRACTION-INDUCED GLUCOSE TRANSPORT

5.1 INTRODUCTION

Previous reported attempts to down-regulate PKc by phorbol esters in muscle have led to mixed results. The treatment of a cultured muscle cell line with TPA resulted in the down-regulation of PKc as assayed by lysine-rich histone (histone III-s), but with the retention of full response to insulin (Klip and Ramlal, 1987). In another muscle cell line TPA treatment was reported to cause a loss of PKc histone kinase activity and loss of TPA effects on glucose transport, but PKc-dependent vinculin phosphorylation by type II isozyme was retained. This latter observation appeared to explain the continued effectiveness of both insulin and DAG in stimulating glucose transport in the "down-regulated" cells (Cooper *et al.*, 1989). In a third study involving isolated mouse soleus muscle down-regulation of PKc, as assayed with lysine-rich histone, inhibited the responses to both TPA and insulin (Tanti *et al.*, 1989).

In the present study isolated soleus muscles were incubated with TPA for 12 h and the response of the muscles to electrical stimulation, as well as insulin, was assessed. PKc activity was monitored by using lysine-rich histone as substrate and phorbol ester binding.

5.2 MATERIALS

3-O-Methyl[1-³H]glucose, [20(n)-³H]phorbol 12,13-dibutyrate and [U-¹⁴C]sucrose were obtained from Amersham (Bucks., U.K.). TPA, PDD, as well as coenzymes, enzymes and substrate were obtained from Sigma (St. Louis, MO, U.S.A.). Insulin, Actrapid MC was from Novo Industri A/S Copenhagen (Denmark).

5.3 METHODS

5.3.1 PREPARATION AND STIMULATION OF THE RAT SOLEUS MUSCLE IN VITRO

Female hooded Wistar rats (55-65 g) were anaesthetised with pentobarbital interperitoneally (5 mg). The solei were removed and attached via the tendons to stainless steel U-shaped supports (15 mm in width) designed to maintain a tension of approximately 10 g (Fig. 4.2). Muscles were incubated with shaking (100 oscillations/min) in pairs at 30°C in stoppered 50 ml flasks which contained 5 ml of incubation medium. The incubation medium consisted of modified Krebs-Ringer saline-bicarbonate buffer (93 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 0.05 mM Na₂ EDTA, 1.27 mM CaCl₂, 5 mM glucose and 25 mM NaHCO₃) gassed with 95% O₂-5% CO₂ to give a pH of 7.4 and contained 0.1% freeze-dried serum obtained from healthy rats. Also included were streptomycin (100 units/ml) and penicillin (100 units/ml)

to stop any bacterial growth during the incubations. The total glucose concentration was adjusted to 5 mM; other additions as indicated were 35 mM mannitol, 100 μ U/ml insulin, PDD (in dimethyl sulphoxide), and TPA (in dimethyl sulphoxide) or dimethyl sulphoxide. The flasks were continuously gassed with 95% O₂-5% CO₂ and the medium was changed every 2 h.

Muscles were incubated for various times then removed from the flasks and washed in 25 ml insulin-free medium before assessing glucose uptake rate, metabolite contents, PKc activity and phorbol ester binding.

5.3.2 DETERMINATION OF GLUCOSE UPTAKE

For glucose uptake determination, solei were remounted by the tendons in a muscle suspension apparatus similar to that described by Nesher *et al.* (1980) (Fig. 4.2). The incubation medium (4 ml) was modified Krebs-Ringer-bicarbonate buffer of the following composition: 93 mM NaCl, 5 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 0.05 mM Na₂ EDTA, 1.27 mM CaCl₂, 25 mM NaHCO₃, 5 mM HEPES, 5 mM pyruvate, 50 mM 3-O-methyl[1-³H]glucose (10 μ Ci/ml) and 5 mM [U-¹⁴C]sucrose (3.2 μ Ci/ml). The medium was gassed 95% O₂-5% CO₂ and the pH was adjusted to 7.4. Gas flow was broken into small bubbles to provide adequate mixing of the incubation media. Two muscles were mounted simultaneously and details for electrical stimulation (Hugo Sachs Elektronik Nerve Muscle Stimulator Type 214) were as follows: 200

ms train of impulses (each impulse 1 ms duration) at a frequency of 50 Hz every 2 s and the voltage was adjusted (10-20 V) to attain full fibre recruitment. Tension development was recorded using a Harvard Bioscience Isometric Transducer. Solei were frozen (liquid N₂) and then homogenised in 2 ml H₂O (Ultra-Turrax, 2 X 30 s), centrifuged (15 min, 220 X g) and 1.5 ml of the supernatant was added to 12 ml ASC II (Amersham) for counting. Inclusion of [U-¹⁴C]sucrose in the incubations enabled correction for the extracellular space.

5.3.3 DETERMINATION OF METABOLITE CONTENT

ATP, ADP, AMP, creatine phosphate and lactate were determined in neutralised perchlorate extracts of frozen muscle. The adenine nucleotides were determined by HPLC (Waters) using a Pharmacia Mono Q anion exchange column. AMP, ADP and ATP were eluted in order using a linear gradient from 4.5 to 150 mM ammonium sulphate in 20 mM Tris-HCl pH 8.0 and detected at 254 nm. Creatine phosphate, lactate and glycogen were determined using standard methods (Bergmeyer, 1974).

5.3.4 DETERMINATION OF PKc ACTIVITY

For PKc determinations, 4 solei were homogenised in 4 ml ice-cold 20 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose and 1 mM dithiothreitol using an Ultra-Turrax homogeniser (2 X 30 s). The homogenate was centrifuged (1 h, 100,000 X g) and the resulting pellet (particulate fraction) extracted (1 h) with 6 ml of 20 mM Tris-HCl buffer, pH 7.5, containing 2.0% (v/v) Triton X-100, 2 mM EDTA, 10 mM EGTA and 1 mM dithiothreitol. The supernatant was collected by centrifugation (30 min, 100,000 X g). Partial purification of PKc and measurement of the enzyme activity was performed as previously described (Chapter 2).

5.3.5 PHORBOL ESTER BINDING STUDIES

[20(n)-³H]Phorbol 12,13-dibutyrate binding was determined by the filtration method (Uchida and Filburn, 1984). Muscle homogenates were processed for the partial purification of PKc (Section 2.3.3). Fractions from the DE-52 column containing PKc activity were pooled from the particulate material and concentrated to approximately 1 ml using Amicon concentrating system (YM30 membrane). Phorbol ester binding reaction mixtures contained 25 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 1.4 mM CaCl₂, 0.4 mM EGTA, 0.4 mM EDTA, 4

mg/ml bovine serum albumin, 100 μ g/ml phosphatidylserine, 20 nM [20(n)- 3 H]phorbol 12,13-dibutyrate \pm 3 μ M TPA in a total volume of 400 μ l in glass tubes (10 X 75 mm). After the addition of 400 μ l of concentrated sample, the tubes were incubated overnight at 4°C. Bound [20(n)- 3 H]phorbol 12,13-dibutyrate was separated from free [20(n)- 3 H]phorbol 12,13-dibutyrate by adding 1 ml of 20 mM Tris-HCl pH 7.5, 10 mM magnesium acetate, 1 mM CaCl_2 and filtering the mixture through 2.4 cm Whatman GF/C filters by suction. The tubes and filters were washed five times with 1 ml of filtering solution, dried in air and then counted for radioactivity. Specific binding was calculated as total binding minus non-specific binding observed in the presence of 3 μ M TPA.

5.4 RESULTS

5.4.1 EFFECT OF LONG-TERM TREATMENT WITH TPA ON INDICES OF VIABILITY

Extended incubation of isolated solei may lead to loss of viability, and agents such as those used in the present study (TPA and DMSO) could exacerbate this effect. Important considerations in minimizing loss of function included muscle size (Newsholme *et al.*, 1986; Maltin and Harris, 1986), incubation temperature and medium composition. Several of these were varied and the following indices of viability determined: water content, ATP, ADP, AMP, creatine phosphate, lactate and glycogen. Indices of incubated muscles were compared with those of freshly excised solei. Components of the incubation medium found to be protective were rat serum, mannitol, insulin and glucose. It was also noted that muscles were best protected when incubated at 30°C. Table 5.1 compares freshly excised solei with muscles incubated for 12 h at two concentrations of TPA and two concentrations of vehicle. Even the highest concentration of TPA used (5 μ M) did not significantly affect any of the metabolites or the water content of isolated solei.

TABLE 5.1 Adenine nucleotides, creatine phosphate, lactate and glycogen content of isolated solei

Treatment	n	Water content ($\mu\text{l/g}$ wet wt)	Metabolite concentration ($\mu\text{mol/g}$ dry wt)					
			ATP	ADP	AMP	Creatine phosphate	Lactate	Glycogen
None*	3	826.4 ± 8.0	21.6 ± 1.3	4.1 ± 1.1	1.0 ± 0.1	39.2 ± 2.0	11.5 ± 0.6	98.1 ± 21.1
0.06% DMSO	3	853.6 ± 8.1	23.6 ± 3.5	3.1 ± 0.5	1.9 ± 0.1	40.6 ± 2.0	16.0 ± 2.2	82.6 ± 7.2
1 μM TPA	3	840.2 ± 11.8	18.1 ± 0.8	2.4 ± 0.2	1.2 ± 0.3	41.0 ± 3.8	13.2 ± 2.5	84.7 ± 4
0.3% DMSO	3	788.1 ± 17.3	21.7 ± 1.1	3.3 ± 0.4	1.1 ± 0.1	38.7 ± 1.4	13.18 ± 1.7	90.5 ± 8.6
5 μM TPA	3	821.4 ± 8.5	20.6 ± 2.4	3.3 ± 0.5	1.0 ± 0.2	41.0 ± 1.2	17.35 ± 1.5	93.0 ± 16.1

Solei were either freshly excised* or had been incubated at 30°C for 12 h in buffered-serum containing 12-0-tetradecanoyl phorbol-13 acetate (TPA), in dimethyl sulphoxide (DMSO) or DMSO alone. Muscles were then removed for the determination of water content (by drying at 110°C for 15 h), and metabolites. Glycogen is expressed as μmol of glucose. Means \pm S.E. are shown. n, number of observations.

5.4.2 EFFECT OF LONG-TERM TREATMENT WITH TPA ON INSULIN- AND CONTRACTION-INDUCED 3-O-METHYL GLUCOSE TRANSPORT

Table 5.2 shows that freshly isolated solei responded to insulin and electrical stimuli by increasing the rate of 3-O-methyl glucose uptake. For insulin (10 mU/ml), the rate increased by 70% from 160 to 272 nmol/min per g and for electrical stimulation the rate increased by 89% to 302 nmol/min per g. The magnitude of these responses was less than noted for soleus muscle in perfused hindlimb (Richter *et al.*, 1984) or *in vivo* (James *et al.*, 1985) but similar to studies *in vitro* (Crettaz *et al.*, 1980; Tanti *et al.*, 1989).

Muscles incubated for 12 h with no additions, 0.06% DMSO or 0.3% DMSO each responded to insulin and electrical stimulation to the same extent as freshly excised solei. Treatment with TPA resulted in a partial loss of response to insulin. For 1 μ M TPA the insulin response decreased from 61% to 40% and for 5 μ M from 57% to 24%. At the higher concentration of TPA the apparent loss of insulin response may be exaggerated due to the increased basal uptake rate. As shown in Table 5.2 the basal uptake rate increased from 189 ± 11 to 198 ± 10 (1 μ M TPA) and 225 ± 6 (5 μ M TPA).

Treatment with 5 μ M TPA for 12 h markedly inhibited the response to electrical stimuli (Table 5.2). At 1 μ M TPA the response decreased from 60% to 31% and at 5 μ M TPA from 56% to zero, when compared to the corresponding DMSO controls. Treatment with 5 μ M PDD, a non-active phorbol ester had no effect on contraction-induced 3-

TABLE 5.2 **Effect of pretreatment of solei with phorbol ester on insulin- and contraction-induced 3-O-methyl glucose transport**

Pretreatment	[³ H]30MG Uptake (nmol/min per g)		
	No. addition	10 mU/ml Insulin	Contractions (30 min)
None [†]	160 ± 6 (3)	272 ± 23 (4)*	302 ± 14 (3)*
Incubated for 12 h	189 ± 11 (4)	303 ± 29 (4)*	283 ± 22 (3)*
0.06% DMSO	187 ± 14 (4)	301 ± 19 (4)*	300 ± 22 (4)*
1 μM TPA in 0.06% DMSO	198 ± 10 (5)	278 ± 7 (5)*	259 ± 15 (5)*
0.3% DMSO	185 ± 17 (5)	291 ± 16 (5)*	288 ± 10 (5)*
5 μM TPA in 0.3% DMSO	225 ± 6 (5)	279 ± 8 (5)*	211 ± 10 (5) n.s.
5 μM PDD in 0.3% DMSO			297 ± (4)*

Solei were either freshly excised[†] or had been incubated at 30°C for 12 h in buffered-serum containing TPA in DMSO, PDD in DMSO or DMSO alone. Muscles were then transferred to a muscle suspension apparatus containing [³H]30MG and [U-¹⁴C]sucrose for an additional 30 min. Uptake of [³H]30MG was determined at 37°C as described in the text and means ± S.E. are shown with the number of observations given in parentheses.

*P<0.05, [³H]30MG uptake was greater than corresponding 'no addition' control. n.s., not significantly different from corresponding 'no addition' control.

O-methyl glucose uptake (Table 5.2).

5.4.3 EFFECT OF LONG-TERM TREATMENT WITH TPA ON CONTRACTION-INDUCED TWITCH TENSION AND GLYCOGENOLYSIS

Since long-term exposure of solei to TPA could produce a variety of changes in muscle biochemistry some of which could be non-specific it was considered important to assess the effects on responses related to glucose uptake. Table 5.3 shows the effect of incubation for 12 h at 30°C on the twitch tension developed by solei which were subsequently stimulated to contract. Incubation for 12 h without addition showed no loss of contractile performance. TPA did not have any significant effect when compared to the corresponding DMSO control (Table 5.3). Thus electrical stimulation of solei previously exposed to a concentration of TPA sufficient to lead to a total loss of glucose transport, responded normally in terms of twitch tension development.

Table 5.1 shows that incubation of muscles for 12 h with or without TPA did not alter glycogen content providing glucose was present in the medium. If the muscles were then stimulated to contract for 30 min the glycogen content decreased markedly from approximately 88 to 12.1 ± 4.4 (n=3) and 16.0 ± 4.8 (n=3) $\mu\text{mol/g}$ dry weight for 0.3% DMSO- and 5 μM TPA-treated muscles, respectively. Thus loss of glucose transport was not associated with a loss of the glycogenolytic response to muscle contractions.

TABLE 5.3 Effect of pretreatment of solei with 12-0-tetradecanoyl phorbol-13 acetate on electrical stimulation-induced twitch tension

Pretreatment	Twitch Tension (g)
None [†]	10.3 ± 1.0 (5)
Incubated for 12 h	9.2 ± 1.5 (4)
0.05% DMSO	8.9 ± 1.2 (7)
1 µM TPA in 0.06% DMSO	8.5 ± 0.5 (5) n.s.
0.3% DMSO	8.0 ± 1.5 (3)
5 µM TPA in 0.3% DMSO	8.3 ± 1.2 (4) n.s.

Solei were either freshly excised[†] or had been incubated at 30°C for 12 h in buffered-serum containing 12-0-tetradecanoyl phorbol-13 acetate (TPA) in dimethyl sulphoxide (DMSO) or DMSO alone. Muscles were then transferred to a muscle suspension apparatus at 37°C and twitch tension recorded during a 30 min period of electrical stimulation (200 ms train of 1 ms impulses at 50 Hz, every 2 s; the voltage was adjusted to attain full fibre recruitment).

Means ± S.E. are shown with the number of observations given in parentheses.

n.s., not significantly different from corresponding DMSO control.

5.4.4 EFFECT OF LONG-TERM TREATMENT WITH TPA ON PKc ACTIVITY AND PHORBOL ESTER BINDING

Long-term treatment of cells has been routinely used to "down-regulate" PKc (Young *et al.*, 1987). Thus in the present study muscles treated with 1 and 5 μ M TPA and which showed loss of contraction-induced glucose transport were assayed for PKc activity. Two methods were used. The first was based on the conventional assay where lysine-rich histone (histone III-s) was used as substrate (Kikkawa *et al.*, 1982). Fig. 5.1 shows that exposure of solei to 1 and 5 μ M TPA for 12 h reduced total (cytosolic + particulate) activity of PKc by approximately 70 and 90% respectively. In the second method total binding sites for [3 H]PDBu were determined. Exposure of solei to 5 μ M TPA for 12 h resulted in a decrease in binding from 2.68 ± 1.11 (n=4; 0.3% DMSO) to 0.81 ± 0.28 (n=4) pmol/g wet weight.

5.5 DISCUSSION

The key finding in the present study was that muscles exposed to TPA for 12 h responded comparably to appropriate controls in terms of tension development and glycogen breakdown but failed to respond in terms of glucose transport. Since long-term exposure to TPA is now a well accepted procedure for down-regulation of PKc it appears likely that the accompanying loss of PKc from the solei was responsible for the loss

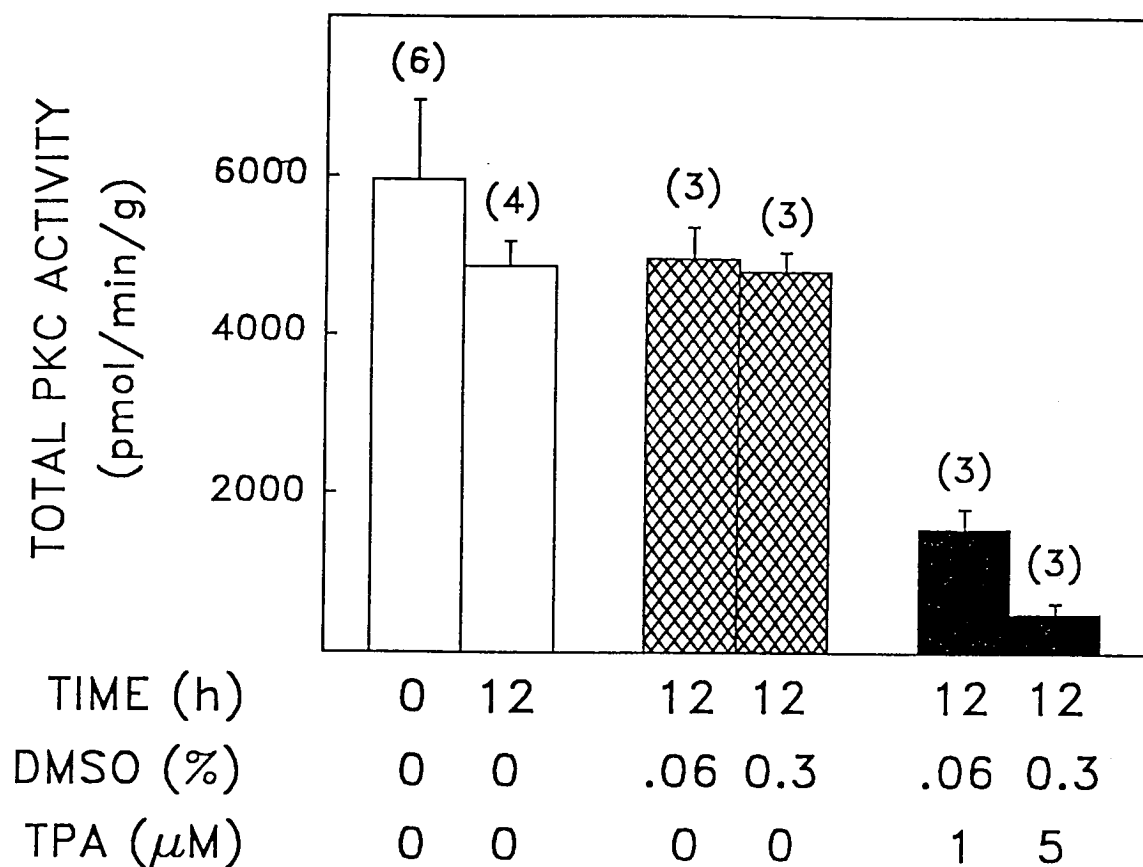


FIG. 5.1. EFFECT OF PRETREATMENT WITH TPA ON TOTAL PKc ACTIVITY. Solei attached to metal supports were incubated at 30°C for 12 h in buffered serum containing TPA in DMSO or DMSO alone, as indicated. The muscles were then homogenized and the total activity (cytosolic + particulate) of PKc was determined. Means \pm S.E.M. are shown, with the number of observations in parentheses. * $P < 0.01$, activity of TPA-treated muscles was less than corresponding DMSO control.

of contraction-induced glucose transport. Indeed inspection of the data of Table 5.2 and Fig. 5.1 shows that down-regulation of PKc by 70% coincided with a loss of approximately 50% of the contraction-induced response, and at higher doses of TPA when PKc was down-regulated further (by 90%) a total loss occurred. However the conclusion that PKc is involved in the metabolic adjustment to muscle contraction, particularly glucose transport must be treated with caution. There are now several identified isozymes of PKc (Schaap *et al.*, 1989) which have different substrate specificities and which may be differentially down-regulated by TPA treatment (Farese *et al.*, 1985; Ase *et al.*, 1988; Cooper *et al.*, 1989). Thus TPA-treatment of KM3 cells has been reported to cause translocation and depletion of type II PKc more quickly than type III enzyme, although both subspecies were totally depleted in 2 h (Ase *et al.*, 1988). Perhaps more importantly when vinculin was used as substrate for the determination of PKc activity (Cooper *et al.*, 1989), TPA treatment of BC3H-1 myocytes did not deplete type II enzyme even though this subspecies was undetectable with histone in the same TPA-treated cells. Thus in the present study it is possible that the apparent down-regulation of PKc as determined by histone III-s kinase activity (Fig. 5.1) did not reflect down-regulation of all subspecies. To address this issue we have made use of the observations by others that skeletal muscle contains predominantly types II and III (Yoshida *et al.*, 1988) and that these types bind [3 H]PDBu with similar affinity (Huang *et al.*, 1988). Clearly, the marked loss of total [3 H]PDBu binding which accompanied the 12 h treatment of solei with 5 μ M TPA would support the view that down-

regulation of both types II and III has occurred.

A further consideration is that the loss of contraction-mediated glucose uptake results not from down-regulation of PKc but from the 12 h period of exposure to TPA as a PKc activator. Sowell *et al.* (1988) have shown that treatment of solei with TPA leads to a marked inhibition of glycogen synthesis and there is some indication in the present study and from others (Klip and Ramlal, 1987) that the basal rate of glucose transport is increased by prolonged TPA exposure. In addition the observation that short-term exposure by phorbol esters induces symptoms of myotonia in skeletal muscle by lowering chloride conductance (Brinkmeier and Jockusch, 1987) suggests that ion channels may be modified by PKc activator. Together these changes may be representative of a general metabolic and functional adjustment to chronic PKc activation which could include loss of contraction-induced glucose transport.

The partial loss of insulin response (Table 5.2) as a result of long-term treatment with TPA might also suggest that PKc is involved, at least in part, in the action of insulin. This would be consistent with a recent report by Tanti *et al.* (1989) but with some difference. In their studies it was reported that 24 h exposure of isolated mouse solei to 1 μ M TPA led to a 40% loss of cytosolic PKc activity and a total loss of insulin-mediated 2 deoxyglucose uptake. However differences between the species may be contributory. For example, 2-deoxyglucose uptake by mouse solei is stimulated by TPA (Tanti *et al.*, 1989). For rat solei 2-deoxyglucose uptake is not affected by 0.04-1.6 μ M TPA (Sowell *et al.*, 1988). Similarly

stimulated by TPA (Tanti *et al.*, 1989). For rat solei 2-deoxyglucose uptake is not affected by 0.04-1.6 μ M TPA (Sowell *et al.*, 1988). Similarly in this laboratory 3-O-methylglucose uptake by isolated rat solei was not stimulated by acute exposure to 1 μ M TPA [117 ± 10 nmol/min per g (n=5) versus 127 ± 21 nmol/min per g (n=7) for control muscles] or 200 μ M 1,2-dioctanoyl-rac-glycerol [79 ± 2 nmol/min per g (n=3) versus 96 ± 16 nmol/min per g (n=3) for matched control muscles].

Comparison of skeletal muscle with other tissues suggests that PKc activity is low (Nishizuka *et al.*, 1984) and may be rendered functionally inoperative by the accompanying presence of relatively large amounts of an endogenous inhibitor of PKc (McDonald *et al.*, 1987). However when assayed under comparable conditions the level of PKc and the cyclic AMP-dependent protein kinase (PKa) are similar in muscle (Nishizuka *et al.*, 1984) and therefore PKc may have as great a significance to muscle metabolism as does PKa.

Finally the link between muscle contraction and glucose transport is still far from clear. Even though this and previous studies described in Chapter 3 imply a role for PKc and generally support an association between phosphoinositol metabolism and excitation-contraction coupling (Volpe *et al.*, 1985; Nosek *et al.*, 1986) it will require further studies to establish that PKc is an activator of glucose transport. There have been a number of recent reports (Hirshman *et al.*, 1988; Douen *et al.*, 1989; 1990; Goodyear *et al.*, 1990c) indicating that acute exercise increased the number of plasma membrane glucose transporters in rat skeletal muscle. This may result from translocation of glucose

transporters from a microsomal fraction to the muscle sarcolemma and therefore be similar to insulin (Klip *et al.*, 1987; Goodyear *et al.*, 1990c) or may result from changes in the intrinsic activity of the glucose transporter (Goodyear *et al.*, 1990c). However, it is likely that the mechanisms involved in insulin and exercise differ in some respect as the effect of a maximal concentration of insulin on glucose transport was further increased by muscle contraction (Ploug *et al.*, 1987).

FINAL PERSPECTIVE

The data presented in this thesis provide for the first time evidence supporting a role for PKc in contraction-induced changes in glucose transport in skeletal muscle *in vivo*. Initial studies described in Chapter 2 confirmed that the enzyme under study was PKc. It was noted that the proportion of PKc activity in the particulate fraction of the resting gastrocnemius-plantaris-soleus skeletal muscle group was high in our preparations (60%). However, it appears unlikely that the procedure for PKc extraction from the tissue would have been the cause of an artificial change in PKc distribution. The requirement of a high detergent concentration for PKc extraction would suggest that the enzyme was tightly bound or partially integrated in the lipid bilayer and not easily dislodged by mechanical disruption. Evidence on three fronts suggest that PKc is relatively inactive under resting conditions. Firstly, unstimulated skeletal muscle probably contains an insufficient level of DAG in the membrane to initiate PKc activation. Secondly, the activity of PKc may be regulated by lipids besides DAG. For example, sphingosine is a competitive inhibitor of PKc *in vitro*. It binds to the DAG binding site on the enzyme (Hannun *et al.*, 1986) and sphingosine may play a role in suppressing PKc activity in resting skeletal muscle. Thirdly, unstimulated skeletal muscle clearly would have very low levels of intracellular Ca^{2+} . One must conclude therefore that the advantage of having a high content of PKc associated with the cell membrane may

provide a mechanism for rapid extracellular signal transduction through the DAG second messenger system to PKc activation.

The results in Chapter 3 provide evidence that electrical-stimulation can induce PKc translocation, DAG and phosphatidic acid production. The increases in DAG appear to be responsible for the translocation of PKc as DAG production and PKc translocation coincide (1) on a time basis following nerve stimulation; (2) when stimulation is halted; and (3) when frequency is varied. It is likely therefore that PKc is subsequently activated when associated with the plasma membrane and when the ternary complex involving the enzyme, DAG, phosphatidylserine and Ca^{2+} is complete.

According to Vergara *et al.*, (1985) the origin of the DAG produced by electrical stimulation of skeletal muscle is from phosphoinositol breakdown. Data from the present study tend to support that view as fatty acid analysis of the newly released DAG was found to resemble the fatty acids of phosphatidylinositol extracted from the same tissue. However, radiolabelled studies would be required to confirm the source of the DAG. In whole skeletal muscle preparations this is not practical due to the slow turnover rate of phospholipids (unpublished observations). It must also be kept in mind the DAG may be partially derived from phospholipids other than phosphatidylinositol. For example, phosphatidylcholine has been shown to be the source of DAG produced in response to Ca^{2+} -mobilising agonists in hepatocytes (Augert *et al.*, 1989). In addition, it is possible that the DAG results from hydrolysis of both phospholipids, initially from agonist stimulated

phosphatidylinositol breakdown followed by subsequent phosphatidylcholine breakdown. It is now established that activators of PKc, such as TPA, can activate a phosphatidylcholine-specific phospholipase C (Besterman *et al.*, 1986) to cause phosphatidylcholine hydrolysis (Muir and Murray, 1987). Phosphatidylinositol hydrolysis and DAG release could initially activate PKc. PKc may then stimulate phosphatidylcholine-specific phospholipase C. Therefore, DAG release could be maintained in the absence of stimuli and phosphatidylinositol breakdown. This may be the explanation why DAG levels are elevated well after electrical stimulation is removed.

Another possible source of DAG is from *de novo* synthesis from glucose or glycogen. Farese and co-workers found the majority of the insulin induced DAG formation in BC3H-1 myocytes was from phosphatidic acid (Farese *et al.*, 1987). In yet another study looking at the source of insulin induced DAG formation in skeletal muscle it was noted that 20-30% of the DAG increase was due to *de novo* synthesis in soleus muscle and almost all of the DAG produced in extensor digitorum longus muscle (Heydrick *et al.*, 1990). Therefore, it is possible that DAG formation resulting from electrical stimulation may derive from a variety of sources including *de novo* synthesis.

In a recent report Turinsky and co-workers were unable to detect any changes in DAG upon electrical stimulation of their muscle preparations (Turinsky *et al.*, 1990) and thus their findings differ from those reported in this thesis. One possible reason for this discrepancy could be the DAG assay itself which relies on the specificity of DAG

kinase for DAG. As mentioned previously DAG may derive from various sources each with a unique fatty acid composition and it is possible that the DAG kinase used may not detect the species of DAG produced by electrical stimulation in skeletal muscle. However, the latter possibility appears highly unlikely as the enzyme has been shown to be specific for the biologically relevant DAG's (Preiss *et al.*, 1987). Another possibility is that Turinsky and co-workers found DAG levels approximately 10 times greater than those found in this present study in rat gastrocnemius-plantaris-soleus muscle group (see Appendix I) and changes in DAG levels therefore may have been masked. Finally, Turinsky and co-workers made no correction for blood engorgement of muscle during exercise (see section 3.4.1.). This would mean that in fact less muscle tissue was present per unit gram of sampled tissue following exercise and a rise in DAG would be masked. The methodology for DAG determination used in this thesis is re-evaluated in Appendix I.

Previously published reports that have investigated the role of PKc in skeletal muscle glucose transport have used the cultured L6 skeletal muscle cell line (Klip and Ramlal, 1987). The data in Chapter 4 provides indirect *in vivo* and *in vitro* evidence of a correlation between PKc activation and an increase in glucose transport activity. For both the *in vivo* and *in vitro* approaches the production of DAG and the translocation of PKc precede the increase in exercise-induced skeletal muscle glucose transport. Furthermore, the increase in glucose transport required multiple stimuli whereas the translocation of PKc and the production of DAG resulted after only a single stimulus. This may suggest that PKc,

although translocated into the membrane requires the sustained presence of Ca^{2+} for activity and to activate the glucose transport process. In support of this view studies using Polymyxin B, which displaces Ca^{2+} from anionic phospholipid, have been shown to inhibit electrically stimulated glucose transport in rat skeletal muscle (Henriksen *et al.*, 1989a). These findings have important parallels with Ca^{2+} -dependent glucose transport in rat cardiac muscle. Previous research from this laboratory found that the α_1 -adrenergic receptor agonist-stimulated glucose transport in rat heart was Ca^{2+} -dependent (Rattigan *et al.*, 1986). The signal transduction pathway for α_1 -adrenergic receptors and exercise are very similar. Both can cause phosphoinositol hydrolysis (Vergara *et al.*, 1985; Brown and Jones, 1987) resulting in DAG release and subsequent PKc activation.

Post-exercise reversal of glucose transport was not investigated in this thesis so a direct comparison between PKc translocation, DAG levels and glucose uptake rate after exercise could not be determined. However, there is evidence to suggest that exercise-stimulated glucose transport continues for at least 30 min post-exercise and full reversal occurs after approximately 2 hours (Goodyear *et al.*, 1990a). Reversal studies found that the PKc translocation and the DAG levels did not return to normal at 30 min post-exercise. This corresponds to the continued transport of glucose and supports the view that they are related. Studies were not carried beyond 30 min and it would be interesting to see if the translocated PKc and the elevated DAG levels return to basal values in association with glucose transport.

The down-regulation of PKc from skeletal muscle has permitted a more direct assessment of PKc involvement in the activation of the glucose transport system. The loss of contraction-induced increase in glucose transport suggests that PKc may have a direct role in the pathway that connects membrane depolarisation and glucose transport. Interestingly, PKc down-regulation had no effect on insulin induced increase in glucose transport.

Evidence has been recently published which tends to support the main findings of this thesis. Epitrochlearis muscle, when incubated with TPA or phospholipase C, showed a stimulated rate of glucose transport. The effects of phospholipase C and insulin were additive, whereas the effects of phospholipase C and muscle contraction were not additive in their ability to stimulate glucose transport. Furthermore, TPA and phospholipase C caused a 2-fold increase in membrane bound PKc activity (Henriksen *et al.*, 1989b). Furthermore, Goodyear and Thompson (1990b) have shown that prior exercise of rats on a treadmill lead to a clear translocation of PKc to the particulate fraction in red muscle and a lesser but similar trend was evident in white muscle. In the same studies insulin had no effect on the translocation of PKc.

Finally, the results presented in this thesis add to the growing accumulation of evidence suggesting PKc as an important part of the mechanism responsible for mediating exercise-induced changes in glucose transport in rat skeletal muscle. The identification of PKc substrates by either ^{32}P labelling or by using monoclonal antibodies, against phosphoserine and phosphothreonine residues, would be useful

experiments to further uncover the precise niche PKc occupies in the glucose transport activation pathway.

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APPENDIX I**TISSUE CONCENTRATION OF
DIACYLGLYCEROL**

APPENDIX I

As mentioned previously, Turnisky *et al.* (1990) reported DAG levels in rat skeletal muscle preparations to be 10 times greater than found in the studies described in this thesis. In response to these contradictory findings we re-examined the lipid extraction procedure, DAG recoveries, thin layer plate separation, staining method and re-checked our calculations. In addition, the same procedure (see Chapter 3) was used to compare DAG concentration in rat heart, liver, blood and aorta (Fig. I). No discrepancies were found in the DAG levels of resting skeletal muscle to those presented in this thesis. DAG levels in both the rat heart and liver was in close agreement to those values previously published (Fig. II) for hamster hearts (Okumura *et al.*, 1990b) and rat liver (Turinsky *et al.*, 1990). Thus, these results support our initial DAG measurement for skeletal muscle. However, even though the DAG levels for rat liver are similar in our studies and Turinsky's, the skeletal muscle levels differ. Moreover, Okumura *et al.* (1990a) have measured lower DAG levels in rat heart than levels presented in this study. Therefore, there seem to be some discrepancies with the measurement of DAG and it could be due to the differences in the way the tissue is handled or to the method used to detect the DAG.

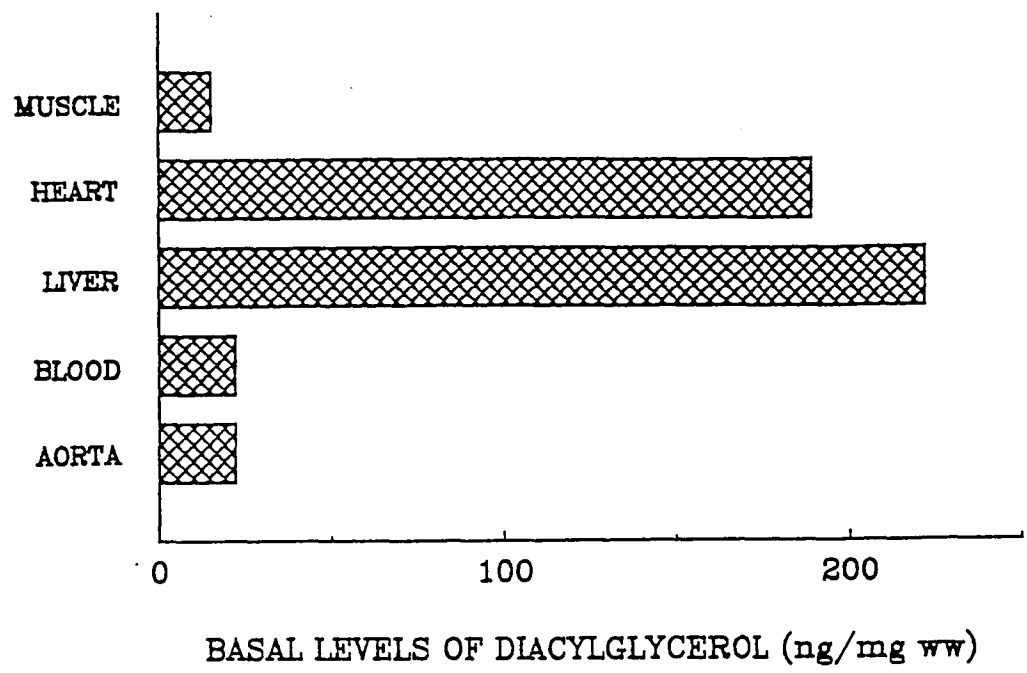


Fig. I. Tissue Diacylglycerol Concentrations. DAG extraction, TLC and laser densitometry measurements was as previously described in section 3.3.3.

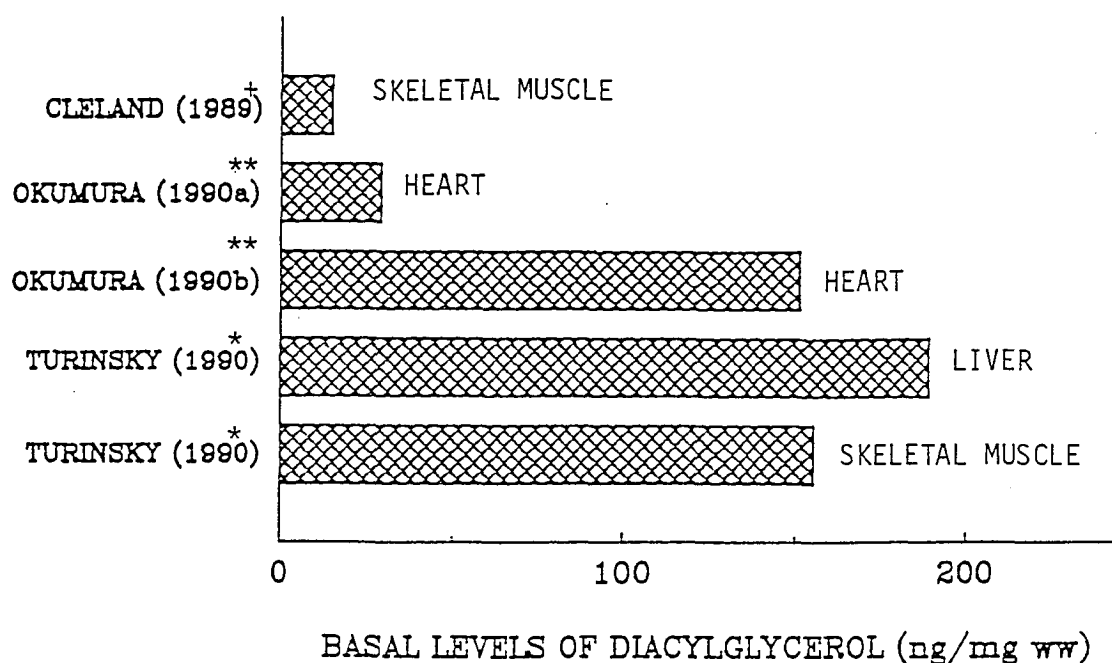


Fig. II. Basal Levels of Diacylglycerol. Methods used were: +, solvent extraction, TLC, Coomassie Blue staining and Laser Densitometry. **, solvent extraction, TLC, Transmethylation and Gas Chromatography. *, solvent extraction, TLC, DAG kinase ³²γ-ATP labelling, TLC and radiolabelled counting of phosphatidic acid spots.

APPENDIX II

**PUBLICATIONS ARISING
FROM THIS THESIS**

Contraction-associated translocation of protein kinase C in rat skeletal muscle

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Electrical stimulation of the sciatic nerve of the anaesthetized rat *in vivo* led to a time-dependent translocation of protein kinase C from the muscle cytosol to the particulate fraction. Maximum activity of protein kinase C in the particulate fraction occurred after 2 min of intermittent short tetanic contractions of the gastrocnemius-plantaris-soleus muscle group and coincided with the loss of activity from the cytosol. Translocation of protein kinase C may imply a role for this kinase in contraction-initiated changes in muscle metabolism.

Ca²⁺ phospholipid-dependent protein kinase; Exercise; Muscle contraction

1. INTRODUCTION

Muscle contraction is associated with changes in the intracellular distribution of Ca²⁺ and changes in several metabolic processes. Recent studies suggest that excitation-contraction coupling in muscle and the resultant mobilization of intracellular Ca²⁺ may involve inositol phosphates [1-4] produced by rapid hydrolysis of phosphatidylinositol. The second product from phosphatidylinositol hydrolysis, diacylglycerol, has been proposed in other tissues to lead to the activation of protein kinase C with translocation of this enzyme from cytosol to the plasma membrane or particulate fraction [5]. Here, we have developed an assay for protein kinase C in skeletal muscle preparations and examined the effect of contraction on the distribution of this enzyme between the cytosol and particulate fractions. A possible role for protein kinase C in exercise-induced Ca²⁺-dependent metabolic changes is discussed.

2. EXPERIMENTAL

2.1. Materials

[γ -³²P]ATP was supplied by Bresa (Adelaide, Australia) and histone IIIs was obtained from Sigma (USA).

2.2. Methods

Fed, male hooded Wistar rats (250 \pm 10 g) were anaesthetized with pentobarbital (12.5 mg). The skin was removed from the left hindlimb and the sciatic nerve exposed and cut to allow positioning of the distal end in a suction electrode. The gastrocnemius-plantaris-soleus muscle group was exposed by careful removal of overlying tissue. The knee was secured by the tibioapatellar ligament and the Achilles tendon was attached via a steel wire to a Harvard Apparatus isometric transducer. Tension development was recorded during electrical stimulation (200 ms trains of 100 Hz applied every 2 s) adjusted (10-20 V) to attain full fibre recruitment. Initial tension was 1065 g which decreased to 600 g after 1 min then remained constant for the remaining 4 min. After 0, 1, 2 or 5 min stimulation the gastrocnemius-plantaris-soleus muscle group (approx. 1.3 g), representing

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mainly fast-twitch red and white muscle [6] was rapidly removed and immediately homogenized in 4 ml ice-cold 20 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose and 1 mM dithiothreitol using an Ultra-Turrax homogenizer (2×30 s). The homogenate was centrifuged for 1 h at $100\,000 \times g$ and the pellet (particulate fraction) extracted twice with 2×6 ml of 20 mM Tris-HCl buffer, pH 7.5, containing 2.0% (v/v) Triton X-100, 2 mM EDTA, 10 mM EGTA and 1 mM dithiothreitol. To the supernatant was added 1/10 vol. of 20 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose, 20 mM EDTA, 5 mM EGTA and 1 mM dithiothreitol. Finely powdered ammonium sulphate was then added to give a concentration of 21% (w/v) and the protein precipitate was removed by centrifugation at $8000 \times g$ for 20 min. A further addition of ammonium sulphate was made to the supernatant [final concentration 45% (w/v)] and the protein pellet, recovered after centrifugation, was dissolved in 0.2 ml of 20 mM Tris-HCl buffer, pH 7.5, containing 2 mM EDTA, 0.5 mM EGTA and 1 mM dithiothreitol. After desalting on a 7 ml column of Bio-Gel P6DG (Bio-Rad, USA) all fractions containing protein were applied to a 3.5 ml column of Whatman DE-52 and eluted using a linear gradient of 36 ml of 0–150 mM NaCl [7]. The Triton X-100 extracts of the particulate fraction were applied directly to DE-52 columns and eluted in a similar manner.

Fractions were assayed for protein kinase C [8] using histone III_s as the substrate and Whatman phosphocellulose P81 paper to collect the acid-precipitable material. Activity was expressed in terms of the weight of the gastrocnemius-plantaris-soleus muscle group from non-stimulated animals (1.3 ± 0.1 g).

3. RESULTS

3.1. Protein kinase C activity in muscle preparations

Published values for protein kinase C in skeletal muscle (373 pmol/min per g [9]) indicated that the activity was considerably less than brain or spleen (17 000–20 000 pmol/min per g [9]) and therefore suggested that difficulties might be encountered in isolating protein kinase C from muscle extracts by anion-exchange chromatography. To overcome this potential problem and to remove some of the contaminating protein, muscle extracts were fractionated by ammonium sulphate and then desalted. By using this approach recovery of protein kinase C was quantitative ($>95\%$) and total activity approached 3500 pmol/min per g.

Fig.1A shows the DE-52 profile of the ammonium sulphate cut (21–45%, w/v) of the cytosolic fractions of non-contracted and contracted (2 min) skeletal muscle. In fig.1B the data for the first and second 2% Triton/EGTA extraction of

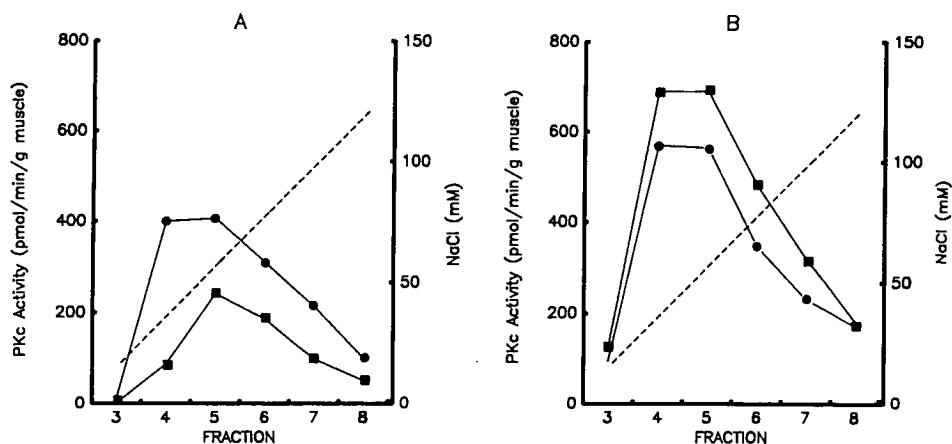


Fig.1. Anion-exchange chromatography of protein kinase C activity from cytosol (A) and particulate (B) preparations of contracting (■) and non-contracting (●) muscle. Details for sample preparation are given in the text. The DE-52 columns were eluted with a linear gradient of NaCl (---). Representative data from five contracting (2 min) and nine non-contracting muscles are shown.

the particulate material have been combined. In general the first Triton/EGTA wash extracted 75% of the particulate activity. From fig.1 it can be seen that contraction led to a decrease in protein kinase C activity in the cytosol and a corresponding increase in activity in the particulate fraction. Both cytosolic and particulate activities were eluted at 40–50 mM NaCl and non- Ca^{2+} /phospholipid-dependent protein kinase activities were negligible (not shown).

Contraction is associated with increased blood flow to the gastrocnemius-plantaris-soleus muscle group and the weight of this group was found to increase from 1.3 to 1.5, 1.6 and 1.6 g at 1, 2 and 5 min, respectively. To rule out the possibility that the increased blood content of the muscle group contributed to the observed translocation of protein kinase C, 0.3 g blood from stimulated animals (cardiac puncture) was added to non-contracted

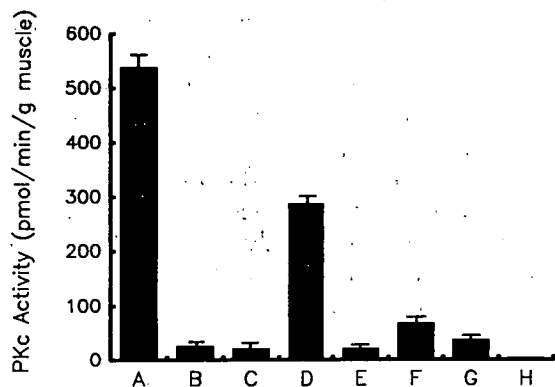


Fig.2. Ligand dependency of protein kinase C from the particulate fraction of contracting muscle. Protein kinase C was partially purified by anion-exchange chromatography of Triton X-100 extracts of the particulate fraction from contracting (2 min) muscles. The complete assay contained 20 mM Tris-HCl, pH 7.5, 0.15 mM EGTA, 0.6 mM EDTA, 7.35 mM Mg acetate, 0.6 mM CaCl_2 , 42 $\mu\text{g/ml}$ L- α -phosphatidyl-L-serine, 0.84 $\mu\text{g/ml}$ diolein, 0.3 mg/ml histone IIIs, 50 μl protein kinase C sample, and 10 μM [γ - ^{32}P]ATP. Changes to the complete assay (A) were: omission of histone (B); omission of phosphatidylserine (C); omission of diolein (D); omission of phosphatidylserine and diolein (E); omission of CaCl_2 with the addition of 7.35 mM EGTA (F); omission of CaCl_2 , phosphatidylserine and diolein with the addition of 7.35 mM EGTA (G); omission of CaCl_2 , phosphatidylserine, diolein and histone with the addition of 7.35 mM EGTA (H). Values are means \pm SE from three experiments.

muscle during homogenization. This had no effect on either the distribution of protein kinase C between the cytosolic and particulate fractions or on the total activity (not shown). Similarly, homogenization of non-contracted muscle in buffer containing 1 mM CaCl_2 also had no effect on these values. It is noteworthy that EGTA could not be included in the initial homogenizing buffers as this displaced the particulate enzyme and diminished

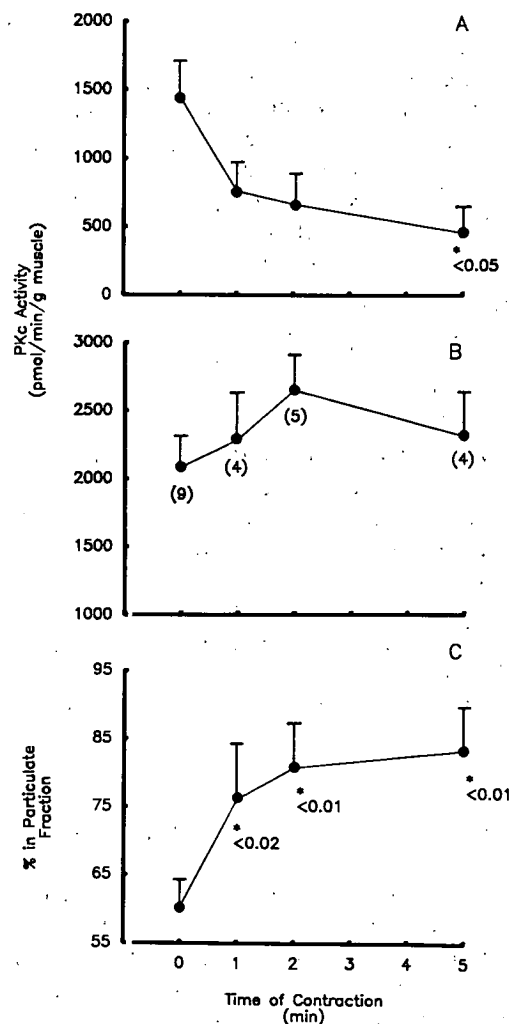


Fig.3. Effect of electrical stimulation-induced contraction of muscle on the distribution of protein kinase C activity in cytosolic (A) and particulate fractions (B). Details are given in the text. Values are means \pm SE with the number of experiments given in parentheses. * P values compared to 0 min using Student's t -test.

the translocation. However, when used in conjunction with 2% Triton X-100, 10 mM EGTA improved the extraction of protein kinase C from the $100\,000 \times g$ particulate material.

3.2. Ca^{2+} and phospholipid dependency of the translocated particulate enzyme

Fig.2 shows the ligand dependency of protein kinase C from the particulate fraction of contracted muscle. Full activity for histone phosphorylation was dependent on the presence of diolein, phosphatidylserine and Ca^{2+} . Omission of these substances as well as histone completely inhibited protein phosphorylation. However when histone alone was omitted some autophosphorylation by endogenous kinase(s) was evident. The data of fig.2 are characteristic of protein kinase C [10,11].

3.3. Time course effects of muscle stimulation

Fig.3 shows the effect of electrically induced contraction of the gastrocnemius-plantaris-soleus muscle group on the distribution of protein kinase C between the cytosolic and particulate fractions. Approx. 60% of the total protein kinase C activity was located in the particulate fraction prior to contraction. This value increased in a time-dependent manner with contraction to reach a maximum of 83% at 2 min with a corresponding decrease in cytosolic activity. After 2 min the loss of activity from the cytosol continued but was not matched by a corresponding stoichiometric increase in the particulate fraction. Approx. 18% of the total activity remained unaccounted for at 5 min.

4. DISCUSSION

Minor modifications to existing methods have enabled quantitative recovery of cytosolic and particulate activities of protein kinase C from skeletal muscle. Presumably because of the removal of endogenous inhibitors, ATP and ATPases, the total activity found was considerably higher than previous reports [9]. For unstimulated muscle approx. 60% of the enzyme was located in the particulate fraction indicating a potentially high state of activation. Clearly this warrants further study.

The present findings represent the first report of contraction-associated translocation of skeletal muscle protein kinase C from the cytosolic to the particulate fraction. These findings extend the

observations of Vergara et al. [1] who reported evidence for inositol 1,4,5-trisphosphate as chemical second messenger between transverse (T)-tubular membrane depolarization and calcium release from the sarcoplasmic reticulum of skinned frog muscle. Thus, T-tubular membrane depolarization may involve stimulation of the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C to form inositol 1,4,5-trisphosphate and diacylglycerol. Subsequent activation of protein kinase C by the latter compound may take place in the presence of Ca^{2+} and phospholipid of the membrane. Phosphorylation of membrane-bound substrates could then account for some of the changes in metabolism which occur as a result of increased muscle contraction. Muscle contraction of the magnitude used in the present studies would be expected to lead to increased glucose transport, glycogenolysis, glycolysis as well as increased oxidative metabolism [12,13]. A likely membrane protein candidate for phosphorylation is the glucose transporter protein which from human red-blood cells is a substrate for brain protein kinase C [14]. Furthermore, activation of protein kinase C in 3T3L1 adipocytes by phorbol esters leads to increased glucose transport as well as phosphorylation of the glucose transporter [15]. However, it is as yet not known whether phosphorylation of this protein by protein kinase C in fact alters its transporter properties.

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Protein Kinase C in Rat Skeletal Muscle

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 MICHAEL G. CLARK

1. INTRODUCTION

Activation of skeletal muscle contraction under physiological conditions involves depolarization of transverse tubular regions of the sarcolemma (Huxley and Taylor, 1958; Hodgkin and Horowicz, 1960; Costantin and Taylor, 1973; Costantin, 1975) leading to Ca^{2+} release from the terminal cisternae of the sarcoplasmic reticulum (Stephenson, 1981). The transverse tubular-terminal cisternae transmembrane signaling mechanism apparently is non-electrical (Donaldson, 1985; Donaldson *et al.*, 1987 and references therein) and there have been numerous studies aimed at examining the chemical triggering of sarcoplasmic reticulum Ca^{2+} release. In this regard some researchers have examined the role of inositol phosphates in the mobilization of intracellular skeletal muscle Ca^{2+} (Volpe *et al.*, 1985; Vergara *et al.*, 1985; Thieleczek and Heilmeyer, 1986; Nosek *et al.*, 1986; Donaldson *et al.*, 1987). Such studies were based on findings that inositol trisphosphate (IP_3) elicited Ca^{2+} release from the endoplasmic reticulum of a wide variety of cells (Berridge, 1981; Suematsu *et al.*, 1984; Hirata *et al.*, 1984; Hokin, 1985). A recent study showed that locally applied microinjected $1 \mu\text{M}$ IP_3 stimulated Ca^{2+} release from peeled skeletal muscle fibers and, although it did not directly activate the contractile apparatus (Donaldson *et al.*, 1987), its role in excitation-contraction coupling was concluded to involve propagation of Ca^{2+} release acting beyond the step of transverse tubule depolarization (Donaldson *et al.*, 1987) at the sarcoplasmic reticulum (Volpe *et al.*, 1985).

An effect of IP_3 to stimulate Ca^{2+} release from, and associated force generation of, skeletal muscle fibers implies that excitation-contraction coupling involves an initial activation of phospholipase C and the production of diacylglycerol. If this is the case, protein kinase C may be activated by translocation from the cytosol to the plasma membrane or particulate fraction (Nishizuka, 1984).

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A subsequent role for protein kinase C in the phosphorylation and regulation of enzymes and processes that adjust in response to increased muscle contraction is possible. The glucose transporter protein from human red blood cells is a substrate for brain kinase C (Witters *et al.*, 1985). In addition, activation of protein kinase C in 3T3L1 adipocytes by phorbol esters leads to increased glucose transport as well as phosphorylation of the glucose transporter (Gibbs *et al.*, 1986). Other substrates that relate to increased glucose transport and are potential candidates for phosphorylation are phosphofructokinase (Hofer *et al.*, 1985) and glycogen synthase (Bouscarel and Exton, 1986).

Thus, in the present study, we have developed an assay for protein kinase C in skeletal muscle preparations and examined the distribution in relation to fiber types and the effect of contraction on the activation of protein kinase C reflected by translocation of this enzyme from cytosol to the plasma membrane or particulate fraction.

2. METHODS

Fed, male, hooded Wistar rats (250 g) were anaesthetized with pentobarbitol (12.5 mg). The skin was removed from the left hindlimb and the sciatic nerve exposed and cut to allow positioning of the distal end in a suction electrode. The knee was secured by the tibioapatellar ligament and the Achilles tendon attached via a steel wire to a Harvard Apparatus isometric transducer. Tension development was recorded during electrical stimulation (200-msec trains of 100 Hz applied every 2 sec) adjusted (10–20 V) to attain full-fiber recruitment. Initial tension was 1065 g which decreased to 600 g after 1 min and then remained constant for the remaining 4 min. After 0, 1, 2, or 5-min stimulation the gastrocnemius-plantaris-soleus muscle group (approximately 1.3 g), representing mainly fast-twitch red and white muscle (Ariano *et al.*, 1973), was rapidly removed and immediately homogenized in 4 ml ice-cold 20 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose, 1 mM dithiothreitol, and 20 μ g/ml trypsin inhibitor (soya bean) using an Ultra-Turrax homogenizer (2 \times 30 sec). The homogenate was centrifuged for 1 hr at 100,000g. The pellet (particulate fraction) was extracted with 30 ml 20 mM Tris-HCl buffer, pH 7.5, containing 2 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 0.2% (v/v) Triton X-100, or with 2 \times 6 ml 20 mM Tris-HCl buffer, pH 7.5, containing 2 mM EDTA, 1 mM dithiothreitol, 2.0% (v/v) Triton X-100, and 10 mM EGTA. To the supernatant was added $\frac{1}{10}$ volume of 20 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose, 20 mM EDTA, 5 mM EGTA, and 1 mM dithiothreitol. Finely powdered ammonium sulfate was added to give a concentration of 21% (w/v) and the protein precipitate was removed by centrifugation at 8000g for 20 min. A further addition of ammonium sulfate was made to the supernatant [final concentration 45% (w/v)] and the protein pellet, recovered after centrifugation, was dissolved in 0.2 ml 20 mM Tris-HCl buffer, pH 7.5, containing 2 mM EDTA, 0.5 mM EGTA, and 1 mM dithiothreitol. After desalting on a 7-ml column of Bio-Gel P6DG (Bio-Rad, USA) all fractions containing protein were applied to a 3.5 ml column of Whatman DE-52 and eluted using a linear gradient of 36 ml of 0–150 mM NaCl (Averdunk and Gunther, 1986). The Triton X-100 extracts of the particulate fraction were applied directly to DE-52 columns and eluted in a similar manner. Fractions were assayed for protein kinase C (Kikkawa *et al.*, 1982) using histone III_s as the substrate and Whatman phosphocellulose P81 paper to collect the acid-precipitable material.

3. RESULTS

3.1. Fiber-Type Distribution of Protein Kinase C

In the course of experiments aimed at recovering protein kinase C activity from skeletal muscle, it became apparent that routine procedures using 0.1–0.3% (v/v) Triton X-100 containing buffers (Kuo, 1980; Kikkawa *et al.*, 1982) were insufficient to fully extract activity associated with the particulate fraction. Thus modification of the extraction procedure was required and buffers containing a higher concentration of Triton X-100 (e.g., 2%) as well as 10 mM EGTA were found to fully extract particulate activity. Table I shows protein kinase C activities in soleus and gastrocnemius white muscles. Soleus muscle contained significantly ($P < 0.01$) more enzyme activity than gastrocnemius white muscle both as total and particulate activity. The percentage of the total contained by the particulate fraction also tended to be greater for the soleus muscle, 73.3 ± 6.1 ($n = 6$) versus 57.6 ± 10.4 ($n = 6$) but was not significant. Furthermore, values for the total content and particulate activity of protein kinase C for the gastrocnemius red and plantaris muscles were less than soleus and more than gastrocnemius white. The percentage of the total contained by the particulate fraction for gastrocnemius red and plantaris muscles were similar to that of the gastrocnemius white (approximately 60%; data not shown). Table I also shows the published values for fiber-type composition (Ariano *et al.*, 1973) and glucose metabolic index values (Rg') for nonexercising and exercising states (James *et al.*, 1985). The latter values were determined for rats *in vivo*. Of the two muscles shown, soleus has the highest content of red slow oxidative fibers and the highest value for Rg' . In addition, the soleus muscle increases its value for Rg' the most during exercise.

3.2. Contraction-Induced Activation of Protein Kinase C

Figure 1 shows the effect of electrically induced contraction of the gastrocnemius-plantaris-soleus muscle group on the distribution of protein kinase C between the cytosolic and particulate fractions. Approximately 60% of the total protein kinase C activity was located in the particulate fraction prior to contraction. This value increased in a time-dependent manner with contraction to reach a maximum of 83% at 2 min with a corresponding decrease in cytosolic activity. After 2 min there was no further loss of activity from the cytosol but the particulate showed some decline. Approximately 13% of the total activity remained unaccounted for at 5 min.

Contraction is associated with increased blood flow to the gastrocnemius-plantaris-soleus muscle group and the weight of this group was found to increase from 1.3 to 1.5, 1.6, and 1.6 g at 1, 2, and 5 min, respectively. To rule out the possibility that the increased blood content of the muscle group contributed to the observed translocation of protein kinase C, 0.3 g blood from stimulated animals (cardiac puncture) was added to noncontracted muscle during homogenization. This had no effect on either the distribution of protein kinase C between the cytosolic and particulate fractions or on the total activity (data not shown). Similarly, homogenization of noncontracted muscle in buffer containing 1 mM CaCl_2 also had no effect on these values. It is noteworthy that EGTA could not be included in the initial homogenizing buffers as this displaced the particulate enzyme and diminished the translocation; however, when used in conjunction with Triton X-100, EGTA improved the extraction of protein kinase C from the 100,000g particulate material.

TABLE I. Comparison of Protein Kinase C Activities, Slow Oxidative Red Fiber Content, and Glucose Metabolic Indices of Soleus and Gastrocnemius White Muscles of Rat

Muscle	Slow oxidative ^a fiber content (%)	Index of glucose ^b metabolism		PKc activity pmol/min per g muscle ^c		
		Rg' rest	Rg' exercise	Cytosol	Particulate	Total
Soleus	84	2.8 ± 0.6	90.4 ± 5.7	804 ± 186	2482 ± 410	3287 ± 426
Gastrocnemius white	5	1.3 ± 0.1	7.0 ± 0.8	602 ± 155	1039 ± 265	1642 ± 220
				n.s.	P < 0.01	P < 0.01

^a Data from Ariano *et al.* (1973).

^b Data from James *et al.* (1985).

^c Values are means ± SE for six animals. Significance of difference between soleus and gastrocnemius white muscle is indicated by *P* values using paired *t* test.

Figure 1 also shows the results of a separate series of experiments where the particulate fraction was extracted using a lower, more conventional concentration of 0.2% Triton X-100. The particulate activity extracted under these conditions increased approximately twofold from 147 ± 28 pmol/min per g muscle to reach a maximum at 2 min and coincided with a corresponding decrease in cytosolic activity of 630 pmol/min per g muscle. The effect of contraction on the percentage content of the particulate fraction was an increase from 11.6 ± 1.2 to $28.0 \pm 4.4\%$ ($P < 0.05$) at 2 min (Fig. 1C). However, while this change reflected a significant translocation of protein kinase C from cytosol to the particulate (membrane) fraction, 76% of the decrease in cytosolic activity remained unaccounted for. As indicated above, this was due to incomplete extraction of the particulate activity. Multiple extractions of the particulate fraction with 0.2% Triton X-100 were unsuccessful (data not shown).

3.3. Characteristics of Skeletal Muscle Protein Kinase C

Since protein kinase C activities noted in the present study were considerably greater than the published value of 373 pmol/min per g muscle (Kuo *et al.*, 1980), it was considered relevant to establish the identity of the enzyme as protein kinase C. Anion exchange chromatography using DEAE-cellulose (Whatman DE-52) indicated that both the cytosolic and solubilized particulate activity eluted at 40–50 mM NaCl (data not shown). In addition, ligand dependency of cytosolic as well as particulate activity was assessed. Table II shows that the properties of the two activities were similar, although the cytosolic activity appeared to be more dependent on diolein. Full activity for histone phosphorylation was dependent on the presence of diolein, phosphatidyl serine, and Ca^{2+} . Omission of these substances as well as histone completely inhibited protein phosphorylation. However, when histone alone was omitted, endogenous protein phosphorylation by endogenous kinase(s) was evident. The data of Table II appear to be characteristic of protein kinase C (Inoue *et al.*, 1977; Takai *et al.*, 1979).

4. DISCUSSION

Three findings emerge from the present study. First, comparison of the activities of protein kinase C in soleus and gastrocnemius white muscle indicates that the former has both a greater total and a greater particulate activity. Also for soleus muscle approximately 73% of the total activity was located in the particulate fraction compared with 58% for the gastrocnemius white muscle.

A greater total content and a greater apparent content of active (particulate) protein kinase C may relate to the metabolic and physiological differences between these two muscle types. Soleus muscle, which is postural, is rich in slow oxidative red fibers (Ariano *et al.*, 1973) and rich in mitochondrial enzymes. The soleus muscle also has a high resting capacity for glucose uptake and metabolism, and also shows a marked increase in glucose uptake during exercise (James *et al.*, 1985). Recent reports that the glucose transporter is phosphorylated by protein kinase C (Witters *et al.*, 1985) and that increased glucose transport coincided with phosphorylation of the transporter in other systems (Gibbs *et al.*, 1986) raise the interesting possibility that control of glucose transport in muscle may be mediated by protein kinase C. The data of Table I lend further support to this view. However, it is not yet known whether phosphorylation of this protein by protein kinase C in fact alters its transport properties.

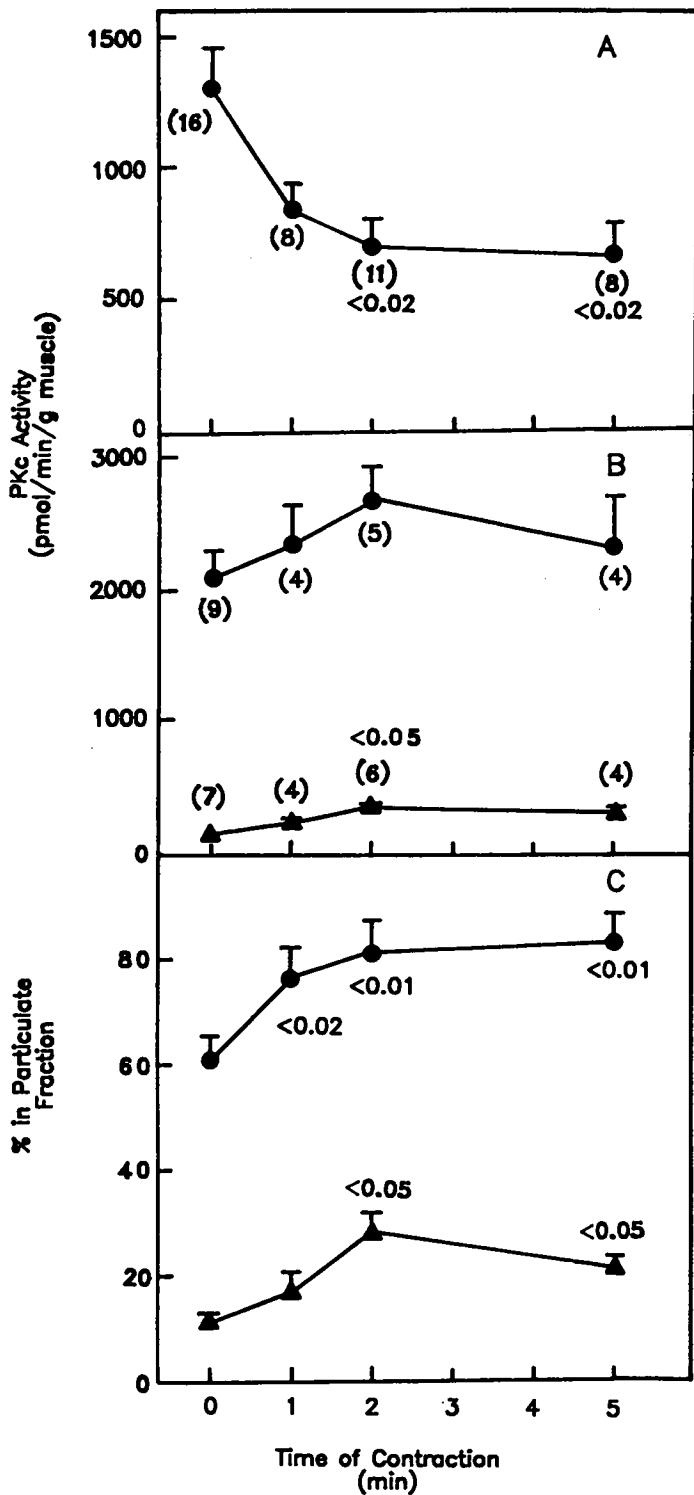


TABLE II. Ligand Dependency of Protein Kinase C from the Cytosolic and Particulate Fractions of Skeletal Muscle

Conditions	PKc activity ^a (pmol/min per g muscle)	
	Cytosolic	Particulate
Complete assay	202 ± 9	530 ± 30
Histone	13 ± 10	27 ± 4
Phosphatidyl serine	15 ± 9	23 ± 7
Diolein	30 ± 4	288 ± 10
Phosphatidylserine, -diolein	10 ± 3	23 ± 3
Ca ²⁺ , + 7.3 mM EGTA	8 ± 1	68 ± 9
Ca ²⁺ , -phosphatidyl serine, -diolein, + 7.3 mM EGTA	5 ± 6	38 ± 6
Ca ²⁺ , -phosphatidyl serine, -diolein, -histone, + 7.3 mM EGTA	1 ± 1	1 ± 1

^a Activity was determined on fractions from DE-52 chromatography. Values shown are means ± SE for three preparations.

The second finding from this study represents the first report of a contraction-associated translocation of skeletal muscle protein kinase C from the cytosolic to the particulate fraction. These findings support the "muscle IP₃ hypothesis" (Volpe *et al.*, 1987) that links phosphatidylinositol turnover with Ca²⁺ release from the sarcoplasmic reticulum. If this hypothesis is correct, phosphorylation of membrane-bound substrates or substrates occasionally associated with particulate material by protein kinase C could then account for some of the changes in metabolism which occur as a result of increased muscle contraction. These include the glucose transporter (Witters *et al.*, 1985; Gibbs *et al.*, 1986), phosphofructokinase (Hofer *et al.*, 1985), and glycogen synthase (Bouscarel and Exton, 1986).

The third finding from this study is that muscle contains a high proportion of its total protein kinase C activity in the particulate fraction. Complete extraction of this activity required relatively high concentrations of Triton X-100 (2%) and EGTA (10 mM). The presence of such a high proportion of the total activity present in what is assumed to be the membrane component implies a high state of activation of this enzyme even under resting conditions. There was no evidence to indicate that the exercise-induced translocated activity could be selectively extracted with lower concentrations of Triton X-100.

Experiments are under way to explain this apparent high state of activation and to determine whether the activity is in fact located in the plasma membrane fraction.

FIGURE 1. Effect of electrical stimulation-induced contraction of muscle on the distribution of protein kinase C activity in cytosolic (A) and particulate (B) fractions. Particulate activity was extracted with buffer containing 0.2% Triton X-100 (▲) or 2.0% Triton X-100 and 10 mM EGTA (●). In part C the distribution of activity in the particulate fraction is expressed as a percentage of the total activity for the corresponding Triton extract. Values are means ± SE with the number of experiments given in parentheses. *P* values compared to 0 min using Student's *t* test are also shown.

5. SUMMARY

Protein kinase C activities in red and white muscles were compared and the effect of electrical stimulation on the distribution of protein kinase C between cytosolic and particulate fractions of the gastrocnemius-plantaris-soleus muscle group was studied. Full extraction of the particulate activity required the combination of high concentrations of Triton X-100 and EGTA. Greater than 60% of the total activity was found to be located in the particulate fraction of resting skeletal muscle suggesting a high state of activation of this enzyme under basal conditions. Soleus muscle, which is rich in slow oxidative red fibers and possesses a high rate of glucose disposal under noncontracting and contracting states, was found to have a twofold greater total activity and a twofold greater particulate activity of protein kinase C than gastrocnemius white muscle. Contraction of the gastrocnemius-plantaris-soleus muscle group by electrical stimulation of the sciatic nerve *in vivo* led to a time-dependent translocation of protein kinase C from the muscle cytosol to the particulate fraction. Maximum translocation of 20% of the total activity occurred after 2 min of intermittent, short, tetanic contractions. It is concluded that protein kinase C of skeletal muscle may play a role in the maintenance of membrane processes in both noncontracting and contracting states.

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PATHOPHYSIOLOGY OF INSULIN ACTION IN MUSCLE

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BIOCHEMISTRY OF INSULIN AND CONTRACTILE EFFECTS IN CARDIAC AND SKELETAL MUSCLE

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INTRODUCTION

Until recently glucose uptake due to muscle contraction was considered to require the presence of a "permissive" amount of insulin (1). Such a relationship implied that muscle contraction led to a markedly increased sensitivity to the prevailing insulin concentration. This now appears not to be the case and the effects of exercise and insulin on muscle glucose transport appear to be quite independent (2) and the mechanism by which contraction stimulates glucose entry into muscle remains to be described. Since excitation-contraction of muscle is associated with mobilization of intracellular Ca^{2+} there has been interest in the possibility that inositol phosphates (3) and as well the Ca^{2+} activated phospholipid-dependent protein kinase (protein kinase C; PKC) may be involved in the metabolic adjustment. Interest in the latter has been heightened by reports that the glucose transporter from human red-blood cells is a substrate for brain PKC (4) and that activation of PKC in a variety of cells by phorbol esters leads to increased glucose transport (5-13). Presently it is uncertain whether PKC is involved in insulin-mediated glucose uptake (6,7,14).

α_1 -Adrenergic effects on heart show similarities to electrical stimulation of skeletal muscle. α_1 -Adrenergic agonists increase contractility (15), activate glucose uptake and transport (16), activate phosphofructokinase (17) and if high doses are used, lead to the activation of glycogenolysis (15). Detailed studies conducted in this laboratory over the last seven years have shown that α_1 -adrenergic control of glucose uptake is Ca^{2+} -dependent (16), cyclic AMP-independent (16), correlates with the activity ratio of phosphofructokinase (17) and involves a discrete receptor-mediated event on glucose transport that can be detected by measuring efflux rates of 3-O-methyl glucose (18) or by D-glucose-inhibitable cytochalasin B binding by isolated sarcolemma (18). In addition high concentrations of perfusate Ca^{2+} simulated all of the changes induced by α_1 -adrenergic agonists where both α_1 -agonists and high Ca^{2+} appear to involve movement of Ca^{2+} inwards across the sarcolemma (18). Insulin appears to increase cardiac glucose transport by increasing the number of glucose transporter proteins in the sarcolemma (18) however the effect of insulin to do so is only partly Ca^{2+} -dependent (18).

The involvement of PKC in the mediation of α_1 -agonist control of cardiac glucose uptake and metabolism is presently unknown but phosphatidyl inositol

turnover with the production of inositol trisphosphate (20) and diacylglycerol (DAG) (21) is linked to α_1 adrenergic receptor activation in this tissue. There has been one report indicating PKC may be involved in insulin-mediated control of glucose transport in heart (13).

With this as background we have investigated the role of PKC in contraction induced increase of muscle glucose uptake.

MATERIALS AND METHODS

Methods for muscle excitation, tension development and for the determination of particulate and soluble PKC activities were as described previously (22). DAG and phosphatidic acid (PA) were determined by thin layer chromatography/Coomassie Blue-laser densitometry.

Glucose uptake was measured using the method of Ferre *et al.* (23) or from the rate of uptake of 3-O-methyl [^3H]glucose (13) into isolated soleus muscles.

RESULTS AND DISCUSSION

Electrical stimulation led to a time-dependent translocation of PKC from the muscle cytosol to the particulate fraction (Fig. 1A). Maximum translocation occurred at 2 min and coincided with a two-fold increase in the concentration of both DAG and PA (Fig. 1B). Time courses for DAG and PA formation as well as for PKC translocation were identical. The time course for the increased glucose transport is shown for electrically-stimulated soleus muscles (Fig. 1C). The rate increased soon after stimulation commenced but did not attain maximum values until approx. 20 min.

In experiments not shown stimulation of muscle *in vivo* for periods of up to 30 min showed no loss of total PKC activity. As well, there was no reversal of PKC translocation nor a decrease in the concentrations of DAG or PA even after 30 min rest following a 5 min period of stimulation. Translocation of PKC was not influenced by variations in applied load to the muscles (0-750 g) at maximal fibre recruitment but as shown in Fig. 2A was dependent on the number of stimuli/min. Contrary to expectations only one stimuli per 30 min was required to attain maximal translocation of PKC (Fig. 2A) and maximum production of DAG and PA (Fig. 2B). Activation of 2-deoxyglucose uptake required more than 35 stimuli over the 30 min period (Fig. 2C).

Therefore it is concluded that electrical stimulation of rat skeletal muscle activates either directly or indirectly the enzymes responsible for the production of DAG and PA. The increases in DAG appear to be responsible for the translocation of PKC from the cytosol to the particulate fraction, as DAG production and PKC translocation coincide (i) on a time basis following nerve

FIG. 1

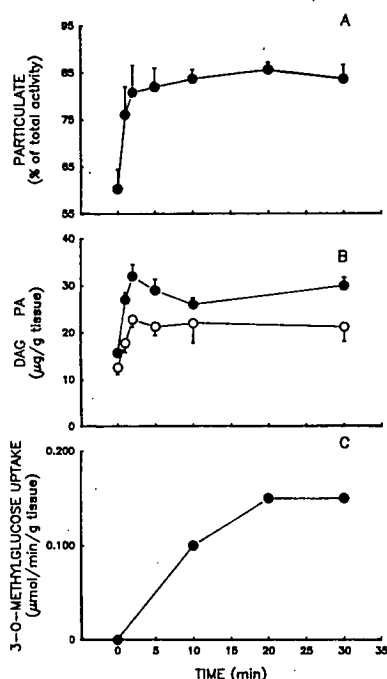


FIG. 2

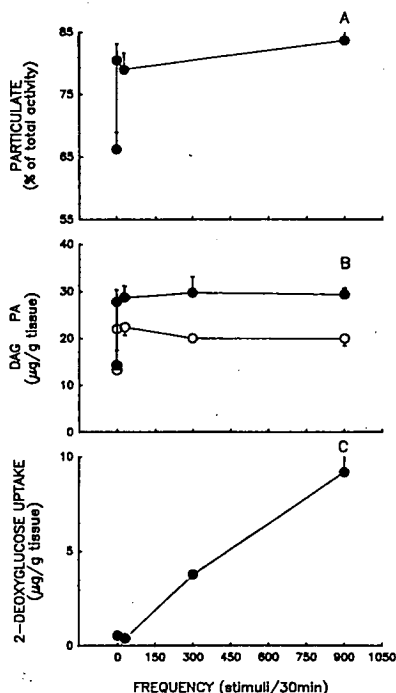


FIG. 1. Effect of electrical stimulation-induced contraction of muscle on the distribution of PKC activity in the particulate fraction and on the formation of DAG (●) and PA (○) *in vivo*. Values are for the gastrocnemius-plantaris-soleus muscle group. 3-O-Methyl glucose uptake was determined using electrically stimulated soleus muscles *in vitro*. Electrical stimulation commenced at $t = 0$ and was set to attain full fibre recruitment (200 ms trains of 100 Hz applied every 2 s, at 10–20 V). Values are means \pm SE for a minimum of $n = 3$.

FIG. 2. Effect of stimuli number on the distribution of PKC activity in the particulate fraction, the formation of DAG (●) and PA (○), and on the uptake of 2-deoxyglucose *in vivo*. Values are for the gastrocnemius-plantaris-soleus muscle group. The sciatic nerve trunk was stimulated to attain full fibre recruitment (200 ms trains of 100 Hz at 10–20 V). Values are means \pm SE for a minimum of $n = 3$.

stimulation; (ii) when stimulation is halted; and (iii) when frequency is varied. The production of DAG and the translocation of PKC precede the increase in muscle glucose transport and these may be causally related. Whereas translocation of PKC and the production of DAG occurs as a result of a single stimulus, glucose transport requires multiple stimuli for activation. This may suggest that PKC, although translocated into the membrane requires the sustained presence of Ca^{2+} to phosphorylate and activate the transport process.

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Exercise-induced Translocation of Protein Kinase C and Production of Diacylglycerol and Phosphatidic Acid in Rat Skeletal Muscle *in Vivo*

RELATIONSHIP TO CHANGES IN GLUCOSE TRANSPORT*

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Contraction-induced translocation of protein kinase C (Richter E. A., Cleland, P. J. F., Rattigan, S., and Clark, M. G. (1987) *FEBS Lett.* 217, 232-236) implies a role for this enzyme in muscle contraction or the associated metabolic adjustments. In the present study, this role is further examined particularly in relation to changes in glucose transport. Electrical stimulation of the sciatic nerve of the anesthetized rat *in vivo* led to a time-dependent translocation of protein kinase C and a 2-fold increase in the concentrations of both diacylglycerol and phosphatidic acid. Maximum values for the latter were reached at 2 min and preceded the maximum translocation of protein kinase C (10 min). Stimulation of muscles *in vitro* increased the rate of glucose transport, but this required 20 min to reach maximum. There was no reversal of translocation or decrease in the concentrations of diacylglycerol and phosphatidic acid even after 30 min of rest following a 5-min period of stimulation *in vivo*. Translocation was not influenced by variations in applied load at maximal fiber recruitment but was dependent on the frequency of nontetanic stimuli, reaching a maximum at 4 Hz. The relationship between protein kinase C and glucose transport was also explored by varying the number of tetanic stimuli. Whereas only one train of stimuli (200 ms, 100 Hz) was required for maximal effects on protein kinase C, diacylglycerol, and phosphatidic acid, more than 35 trains of stimuli were required to activate glucose transport. It is concluded that the production of diacylglycerol and the translocation of protein kinase C may be causally related. However, if the translocated protein kinase C is involved in the activation of glucose transport during muscle contractions, an accumulated exposure to Ca^{2+} , resulting from multiple contractions, would appear to be necessary.

The Ca^{2+} -activated and phospholipid-dependent protein kinase (protein kinase C) has been shown to have widespread distribution in mammalian tissues including skeletal muscle (1). This protein kinase can be activated by a low concentration of Ca^{2+} in the presence of both phosphatidylserine and diacylglycerol (2). In addition, it has been proposed that the

release of diacylglycerol through the signal-induced breakdown of phosphatidylinositol may trigger the activation of protein kinase C *in vivo* (2) reflected by translocation of this enzyme from cytosol to the plasma membrane or particulate fraction.

Since excitation-contraction of muscle is associated with mobilization of intracellular Ca^{2+} , there has been interest in the possibility that inositol phosphates (3-6) as well as protein kinase C may be involved in the accompanying metabolic changes. Among the possible substrates for protein kinase C is the glucose transporter protein, and studies from Lienhard's laboratory have indicated that this protein from human red blood cells is a substrate for brain protein kinase C (7). In addition, activation of protein kinase C in 3T3L1 adipocytes by phorbol esters was shown to increase glucose transport as well as phosphorylation of the glucose transporter (8). Thus, in the present study, we have attempted to extend our previous findings that muscle contraction *in vivo* leads to the translocation of protein kinase C (9). Experiments have focused on the relationships among diacylglycerol production, protein kinase C translocation, and the rate of glucose transport.

EXPERIMENTAL PROCEDURES

Materials

L- α -Phosphatidyl-L-serine, L- α -phosphatidylinositol, L- α -phosphatidylethanolamine, L- α -phosphatidylcholine, tripalmitin, diolein, 1,2-dioleoyl-*sn*-glycerol, dipalmitoyl phosphatidate, histone III-S, HEPES,¹ EGTA, 1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphorylcholine, and phospholipase C (type XII) were obtained from Sigma. [γ - ^{32}P]ATP was purchased from Bresatec (Adelaide, Australia). Silica Gel 60 F254 thin layer chromatography plates were from Merck; DE52 cellulose and P81 phosphocellulose paper were from Whatman, and 2-deoxy[1- ^3H]glucose, [U- ^{14}C]sucrose, and 3-O-methyl[^3H]glucose were from the Amersham Corp.

Methods

Preparation and Stimulation of Skeletal Muscle *in Vivo*—Fed male hooded Wistar rats (250 ± 10 g) were anesthetized with pentobarbital intraperitoneally (12.5 mg). The skin was removed from the left hindlimb, and the sciatic nerve was exposed and cut to allow positioning of the distal end in a suction electrode. The gastrocnemius-plantaris-soleus muscle group was exposed by careful removal of overlying tissue. The knee was secured by the tibioapatellar ligament, and the Achilles tendon was attached via a steel wire to a Harvard Bioscience isometric transducer. Tension development was recorded during electrical stimulation (Hugo Sachs Elektronik nerve muscle stimulator type 214). Unless indicated otherwise, the gastrocnemius-plantaris-soleus muscle group was stimulated by applying 200-ms trains of impulses (each impulse 0.1-ms duration) at a frequency of 100 Hz every 2 s. The voltage was adjusted (10-20 V) to attain full

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¹ The abbreviations used are: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EGTA, [ethylenebis(oxyethylenetri-)]tetraacetic acid.

fiber recruitment. Although the initial tension was set at 500 g, this, as well as frequency of impulse and the number of trains, could be varied. If developed tension during full fiber recruitment decreased to less than 70% of the initial value, the experiment was abandoned. Occasionally this occurred when the dose of anesthetic was too high, and the rat died.

Extraction of Protein Kinase C—After stimulation, the gastrocnemius-plantaris-soleus muscle group (approximately 1.3 g), representing mainly fast twitch red and white muscle (10), was rapidly removed (approximately 5 s) and homogenized in 4 ml of ice-cold 20 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose and 1 mM dithiothreitol using an Ultra-Turrax homogenizer (2 × 30 s). The homogenate was centrifuged (1 h, 100,000 × g), and the resulting pellet (particulate fraction) was extracted (1 h) with 6 ml of 20 mM Tris-HCl buffer, pH 7.5, containing 2.0% (v/v) Triton X-100, 2 mM EDTA, 10 mM EGTA, and 1 mM dithiothreitol. The supernatant was collected by centrifugation (30 min, 100,000 × g). In initial studies (9), the particulate fraction was extracted twice, and the initial supernatant (cytosolic fraction) was fractionated using (NH₄)₂SO₄ precipitation. Omission of the second pellet extraction or (NH₄)₂SO₄ fraction did not significantly affect the total activity recovered (combined contribution 5%).

Supernatant extracts containing protein kinase C were applied to columns (4 ml) of Whatman DE52, which were then washed with 20 ml of 20 mM Tris-HCl, pH 7.5, containing 2 mM EDTA, 0.5 mM EGTA, and 1 mM dithiothreitol. Protein kinase C activities and protein kinase M were eluted using linear gradients of 36 ml of 0–0.15 M NaCl (9) and 100 ml of 0–0.4 M NaCl, respectively. Fractions of 4 ml were collected, and typically protein kinase C activity was found in fractions 4–6 corresponding to 30–90 mM NaCl.

Fractions were assayed for protein kinase C (11) using histone III-S as the substrate and Whatman phosphocellulose P81 paper to collect the acid-precipitable material. Activity was calculated from the difference in ³²P incorporated into histone in the presence and absence of added phosphatidylserine, diolein, and Ca²⁺ and was expressed as pmol of ³²P incorporated/min/g, wet weight, of the excised gastrocnemius-plantaris-soleus muscle group (1.3 ± 0.1 g) from nonstimulated weight-matched animals. One gram, wet weight, of this muscle contained 220 ± 7 (n = 5) mg of protein.

Diacylglycerol and Phosphatidic Acid Determinations—Muscles required for diacylglycerol and phosphatidic acid analysis were initially freeze-clamped *in situ* using liquid N₂-cooled tongs. Powdered frozen tissue (0.5 g) was then extracted (Ultra-Turrax, 2 × 30 s) with 1.2 ml of chloroform:methanol (1:2). Further additions of chloroform (0.4 ml) and H₂O (0.4 ml) were made with vigorous mixing (12). Samples were then centrifuged (5 min, 2000 × g). The chloroform layer was removed and the aqueous layer reextracted five times, as above. The chloroform extracts were filtered through glass wool and the solvent evaporated under N₂. The residue was dissolved in 0.5 ml of chloroform, and an aliquot (20 μl) was applied to a Silica Gel 60 F254 TLC plate. The solvent system for the resolution of diacylglycerol was toluene:diethyl ether:ethanol:ammonium hydroxide (50:40:2:0.2). Plates were stained with Coomassie Blue R-250 (13), and the amount of diacylglycerol (1,2-isomer, *R_F* 0.67) was measured by laser densitometry. Reference standards were diolein (comprising approximately 80% 1,3-dioleoyl-glycerol (*R_F* 0.76) and approximately 20% 1,2-dioleoyl-glycerol (*R_F* 0.67), 1,2-dioleoyl-*sn*-glycerol (*R_F* 0.67), and 1-*O*-hexadecyl-2-acetyl glycerol (*R_F* 0.88)). The intensity of the blue band corresponding to diacylglycerol was proportional to the mass applied to the plate for the range 0.125–1 μg. Standards of 1,2-dioleoyl-*sn*-glycerol (0.125–1 μg) were spotted on each plate and used to quantify the material of identical *R_F* present in tissue extract. Recovery of authentic 1,2-dioleoyl-*sn*-glycerol added to muscle homogenates was greater than 85% using the above procedure.

The solvent system for the resolution of phosphatidic acid was ethyl acetate:isooctane:acetic acid (45:25:10). Plates were stained with Coomassie Blue R-250, and the amount of phosphatidic acid (*R_F* 0.27) was measured by laser densitometry. The reference standard was 0.125–1 μg of dipalmitoyl phosphatidate (*R_F* 0.27). Recovery of authentic material from muscle homogenates was also greater than 85% by this procedure. However, recovery of phospholipids by this method was variable, and hence a different procedure was used.

Phospholipid and Triacylglycerol Extraction—Powdered frozen tissue (0.5 g) was extracted (Ultra-Turrax, 2 × 30 s; vortex 1–2 min) with 1.2 ml of chloroform, methanol, 35% (v/v) HCl (200:100:1, v/v). Further additions of chloroform (0.4 ml) and water (0.4 ml) were made with vigorous mixing. Samples were then centrifuged (5 min, 2000 × g). The chloroform layer was removed and the aqueous layer reextracted five times, as above. The chloroform extracts were pooled

and then evaporated to dryness under N₂. The residue was dissolved in 0.5 ml of chloroform, and an aliquot (100 μl) was applied to a Silica Gel 60 F254 TLC plate. Procedures for the resolution of phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine and for the separation of triacylglycerol were as described by Christie (14).

Fatty Acid Composition—Diacylglycerol-, phosphatidic acid-, phospholipid-, and triacylglycerol-containing fractions were saponified (14) and the free fatty acids converted to their methyl esters (15). Gas-liquid chromatography was performed using a 183 × 0.40-cm (inner diameter) column of 3% Silar 10C on Chromosorb G 80/100 mesh (Alltech Associates Inc.), a Hewlett-Packard model 5890 gas chromatograph, and flame ionization detector. Fatty acid methyl esters were eluted at 180 °C. Peak areas were integrated using a Skimadzu C-R3A Chromatopac reporting integrator.

Glucose Transport and Uptake—Glucose uptake *in vivo* was determined using the method of Ferré *et al.* (16) derived from the technique of Sokoloff *et al.* (17). The theoretical basis of the method is outlined by Ferré *et al.* (16), and the rate of glucose uptake (*R_i*) from the injection of the label (time = 0) until the sampling time (*τ*) in the present study was calculated using their steady-state equation.

$$R_i = \frac{[2\text{-deoxyglucose 6-phosphate}]\tau}{LC^* \int_0^{\tau} (C_B^*/C_B) dt}$$

where *LC* (the lumped constant) is a correction for the discrimination against 2-deoxyglucose in glucose transport and phosphorylation pathways. In the present experiments, the lumped constant was set at 1.0, as the average of the values determined by Ferré *et al.* (16) for soleus (0.95) and extensor digitorum longus (1.05); *C_B*^{*} and *C_B* represent the blood 2-deoxyglucose expressed in terms of radioactivity and the blood glucose concentration, respectively.

Briefly, a bolus injection of 30 μCi of 2-deoxy[1-³H]glucose was administered via a carotid artery catheter into the anesthetized animal. Samples of blood were then removed at intervals of 5 min to monitor the concentrations of both 2-deoxyglucose and glucose. Electrical stimulation of the sciatic nerve immediately followed injection of the labeled 2-deoxyglucose. The gastrocnemius-plantaris-soleus muscle group was excised at steady state (30 min) for the determination of 2-deoxyglucose 6-phosphate concentration (16).

Glucose uptake *in vitro* was determined using isolated soleus muscle (approximately 40 mg) from juvenile male hooded Wistar rats (80–100-g body weight). Muscles were removed from the animal and quickly mounted by the tendons on a muscle suspension apparatus similar to that described by Nesher *et al.* (18). The incubation medium (4 ml) was modified Krebs-Ringer bicarbonate buffer of the following composition: 93 mM NaCl, 5 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 0.05 mM Na₂EDTA, 1.27 mM CaCl₂, 25 mM NaHCO₃, 5 mM HEPES, 5 mM pyruvate, 50 mM 3-*O*-methyl[1-³H]glucose (0.4 μCi/ml), and 5 mM [U-¹⁴C]sucrose (0.125 μCi/ml). The medium was gassed with 95% O₂, 5% CO₂, and the pH was adjusted to 7.4. Gas flow was broken into extremely small bubbles providing thorough mixing of the incubation medium. Two muscles were mounted simultaneously, and details for electrical stimulation were similar to those described above for studies *in vivo* except that impulses were of 1-ms duration. [U-¹⁴C]sucrose was added to enable the extracellular space in the muscle to be measured. The frozen muscles were homogenized in 2 ml of H₂O (Ultra-Turrax, 2 × 30 s), centrifuged (15 min, 220 × g), and 1.5 ml of the supernatant was added to 12 ml of ASC II (Amersham Corp.) for counting.

RESULTS

Protein kinase C has been reported to be present in most tissues including skeletal muscle (1, 22). In Table I, the content of protein kinase C of four different rat muscles is shown as well as the distribution of activity between cytosolic and particulate components. The order of activity was soleus > plantaris > gastrocnemius red = gastrocnemius white. Soleus muscle also had the greatest proportion of activity in the particulate fraction (67%) with plantaris, gastrocnemius white, and gastrocnemius red each having approximately 52%. Since the gastrocnemius-plantaris-soleus group comprises 5% soleus, 12.5% plantaris, 43.7% gastrocnemius white, and 38.7% gastrocnemius red muscle (by weight), the combined data for the muscle group were 2806 pmol/min/g with ap-

TABLE I

Protein kinase C activity in muscles of the rat hindlimb

Muscles were rapidly removed from anesthetized rats and homogenized in 3 volumes of 20 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose and 1 mM dithiothreitol using an Ultra-Turrax homogenizer (2×30 s). The homogenates were centrifuged and the pellets extracted as described under "Experimental Procedures." Protein kinase C activity was measured in fractions following DE52 anion exchange chromatography of the initial supernatant and the solubilized Triton X-100 extracts of the particulate material. Each value represents the mean \pm S.E. of determinations done in duplicate on muscles from six animals.

Muscle	Protein kinase C activity		
	Cytosolic fraction	Particulate fraction	Total
		pmol/min/g	
Soleus	1909 \pm 376	4015 \pm 622	5956 \pm 995
Plantaris	1854 \pm 404	1999 \pm 384	3851 \pm 732
Gastrocnemius white	1121 \pm 111	1249 \pm 170 ^a	2392 \pm 248 ^a
Gastrocnemius red	1236 \pm 177	1304 \pm 178 ^a	2538 \pm 322 ^a

^a Significantly different from soleus ($p < 0.05$).

proximately 53% of the total protein kinase C activity in the particulate fraction. This compares favorably with the total activity for the gastrocnemius-plantaris-soleus muscle group determined as a whole (see Table III and Fig. 1). It is noteworthy that published values for protein kinase C content of skeletal muscle vary between 373 pmol/min/g (1) and 80 pmol/min/mg of protein (22), but the lower value probably reflects the presence of inhibitors and ATPases that are in homogenates of this tissue and that are eliminated by the anion exchange chromatography step.²

The high proportion of total activity associated with the particulate fraction from resting muscle implied that the enzyme was principally located in skeletal muscle sarcolemma. This proved to be the case as a 50-fold purified sarcolemmal vesicle preparation from skeletal muscle (19–21) was found to contain high activities of protein kinase C.³ Thus, contributions to the particulate protein kinase C activity from nonmuscle cells (nerve, mast, endothelial cells, etc.) were considered to be minimal.

Fig. 1 shows the effect of electrical stimulation-induced contraction on the translocation of protein kinase C activity from the cytosol to the particulate fraction. Approximately 60% of the total protein kinase C activity was located in the particulate fraction prior to contraction. This value increased in a time-dependent manner with contraction to reach a maximum of 86% at 10 min with a corresponding decrease in cytosolic activity. Although there was a tendency for the particulate activity to decrease after 10 min (Fig. 1A) while the cytosolic activity remained constant (Fig. 1B), the decrease was not significant. Thus, total activity remained the same throughout the 30-min contraction period, and there was no evidence of protein kinase M formation, as assessed by the appearance of a Ca^{2+} -phospholipid-independent histone kinase eluting later from the DE52 columns.

Muscle contraction *in vivo* is accompanied by increased blood flow due to vasodilation, and the blood content of the muscles increases. Thus, the weight of the gastrocnemius-plantaris-soleus muscle group was found to increase from 1.30 ± 0.05 ($n = 7$) to 1.5, 1.6, and 1.82 ± 0.04 ($n = 6$) at 1, 2, and 5 min, respectively. To rule out the possibility that the increased blood content of the muscle group contributed to the observed translocation of protein kinase C and the increases in diacylglycerol and phosphatidic acid, 0.35 g of blood from

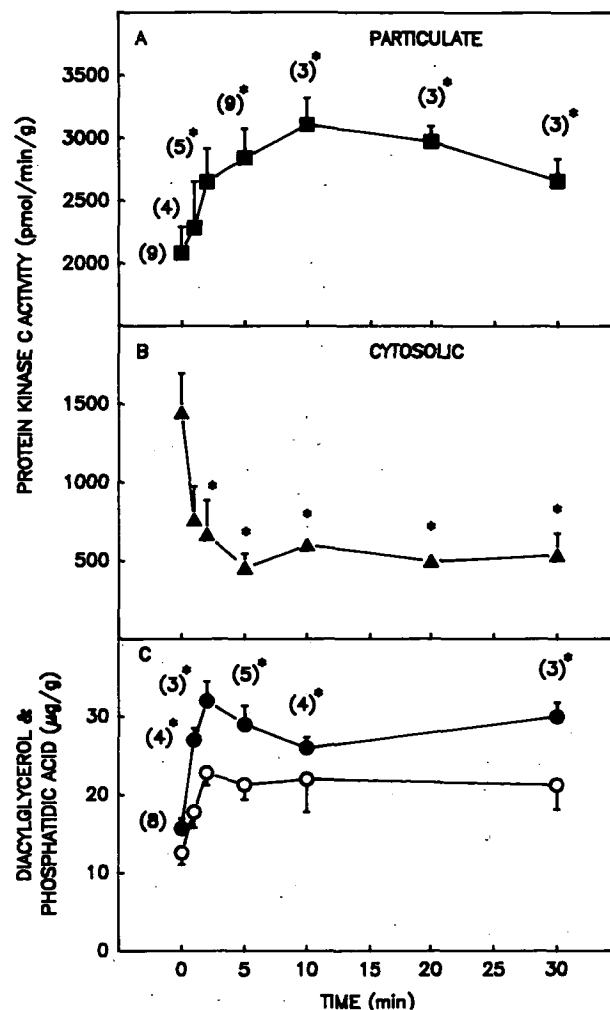


FIG. 1. Time course of the contraction-induced translocation of protein kinase C and formation of diacylglycerol and phosphatidic acid. Rats were anesthetized and the skin of the left hindlimb removed. The sciatic nerve was exposed and cut to allow positioning of the distal end in a suction electrode. The gastrocnemius-plantaris-soleus muscle group was exposed, the knee secured, and the Achilles tendon attached to an isometric force transducer as described under "Experimental Procedures." The sciatic nerve was then stimulated by applying 200-ms trains of impulses (each impulse 0.1-ms duration) at a frequency of 100 Hz every 2 s for the times shown. The voltage was set to attain full fiber recruitment. The muscle group was then rapidly excised and processed as described under "Experimental Procedures" for the determination of protein kinase C activity in the particulate (A) and cytosolic (B) fractions and for the determination of diacylglycerol (●) and phosphatidic acid (○) (C). Each value represents the mean \pm S.E. of determinations done in duplicate with the number of animals shown in parentheses. *, Significantly different from zero time point ($p < 0.05$).

stimulated animals (cardiac puncture) was added to noncontracted muscle during homogenization. This had no effect on any of the above parameters (data not shown).

Fig. 1C shows the effect of muscle stimulation on the production of diacylglycerol and phosphatidic acid. Both substances showed a 2-fold increase in concentration over the first 2 min of electrical stimulation, and maximal levels were then maintained for the remaining 28 min. The apparent trend for diacylglycerol concentrations to decrease after 2 min was not statistically significant.

The fatty acid composition of diacylglycerol and phosphatidic acid from resting and contracting (2 and 30 min) muscles is shown in Table II as well as the fatty acid composition of

² P. J. F. Cleland, S. Rattigan, and M. G. Clark, unpublished results.

³ S. Rattigan, unpublished results.

TABLE II

Fatty acid composition of phospholipids, diacylglycerol, and phosphatidic acid

Muscles were from resting (Non-ex) or electrically stimulated (Ex) gastrocnemius-plantaris-soleus groups *in vivo* and were stimulated for the times shown. Phospholipids, diacylglycerol, and phosphatidic acid were fractionated by thin layer chromatography and collected. Transmethylation and gas-liquid chromatography were as described under "Experimental Procedures." Values are expressed as mol/100 mol of the parent lipid and are the means of three determinations. Abbreviations: PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TG, triacylglycerol.

Fatty acid	Phospholipids from resting muscle				Diacylglycerol			Phosphatidic acid		
	PI	PC	PE	TG	Non-ex	Ex _{2 min}	Ex _{30 min}	Non-ex	Ex _{2 min}	Ex _{30 min}
16:0	31.2	28.3	33.0	46.6	49.3	49.7	48.5	27.5	39.6	41.5
18:0	38.3	9.6	15.6	36.1	21.3	19.4	18.5	38.9	17.8	26.1
18:1		45.3	27.8	12.6						8.8
18:2		7.9	14.7	4.5				26.7	4.8	6.0
20:4	30.4	12.7	17.2	0.4	21.6	25.4	23.0	6.8	37.8	24.4

triacylglycerol, phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine. The diacylglycerol was rich in palmitic acid and contained stearic and arachidonic acids but did not contain either oleic or linoleic acids. In addition, the composition did not differ between resting and contracting muscles (2 and 30 min). The fatty acid composition of phosphatidate from resting muscle showed some resemblance to that of triacylglycerol; however, it did not contain oleate and instead contained an appreciable proportion of linoleic acid. As a result of exercise, differences between triacylglycerol and phosphatidic acid became more evident. For phosphatidic acid, there was a marked increase in the proportions of arachidonate and palmitate with decreases in stearate and linoleate. Thus, a comparison of the fatty acid composition of triacylglycerol with diacylglycerol at 2 and 30 min postexercise and with phosphatidic acid at 2 min postexercise would suggest that triacylglycerol is not the precursor. However, some formation of phosphatidic acid from triacylglycerol may account for the changes in fatty acid composition which are evident at 30 min postexercise (Table II).

Fig. 2 shows the results of experiments intended to examine the effect of a period of rest on muscles following electrical stimulation. Muscles were stimulated for 5 min with a total of 150 trains of tetanic stimuli and then allowed periods of 10, 20, and 30 min of rest before excision. As noted for Fig. 1, electrical stimulation increased the particulate fraction content of protein kinase C (Fig. 2A) and produced a corresponding decrease in the cytosolic fraction content of the enzyme (data not shown). There was also a 2-fold increase in the muscle concentrations of diacylglycerol and phosphatidic acid (Fig. 2B). However, resting the muscle for periods up to 30 min after stimulation did not lead to a reversal of any of these changes.

Three parameters that influence muscle response to electrical stimulation are applied load, frequency of nontetanic stimuli, and the total number of tetanic stimuli. Accordingly, each of these has been varied independently and the effects examined. Table III shows the effect of varying the initial load at maximum fiber recruitment. For these experiments, the isometric force transducer was standardized against weights of 100, 500, and 1000 g, and then positioned so as to apply an initial tension on the gastrocnemius-plantaris-soleus muscle group of 0, 200, 500, or 700 g. The sciatic nerve was then stimulated for a 5-min period with a total of 150 tetanic trains. The data show that translocation of protein kinase C

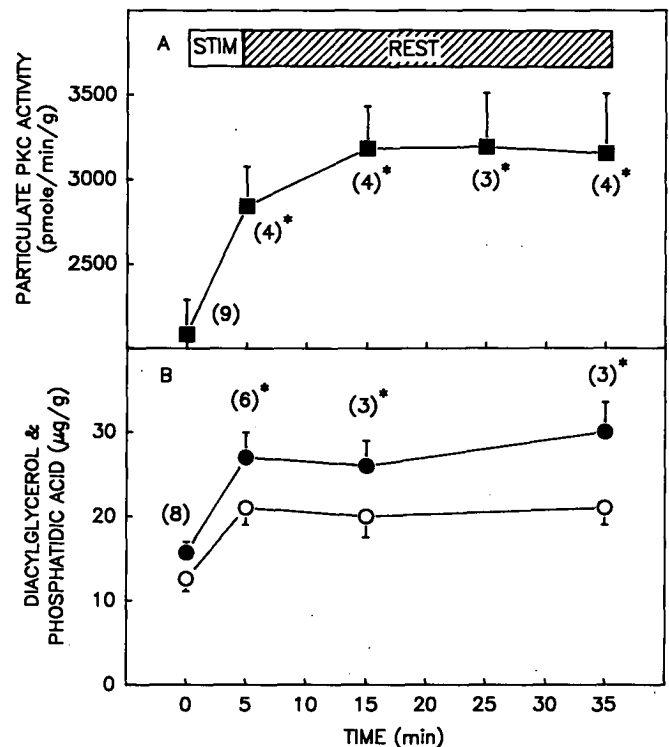


FIG. 2. Effect of stimulation followed by a period of rest on the translocation of protein kinase C (PKC) and formation of diacylglycerol and phosphatidic acid. Rats were anesthetized and the skin of the left hindlimb removed. The sciatic nerve was exposed and cut to allow positioning of the distal end in a suction electrode. The gastrocnemius-plantaris-soleus muscle group was then exposed, the knee secured, and the Achilles tendon attached to an isometric force transducer. The sciatic nerve was stimulated for 5 min followed by a rest period of up to 30 min. At the times shown, the muscle group was excised and processed as described under "Experimental Procedures" for the determination of protein kinase C activity in the particulate fraction (A) and for the determination of diacylglycerol (●) and phosphatidic acid (○) (B). Each value represents the mean \pm S.E. of determinations done in duplicate with the number of animals shown in parentheses. *, Significantly different from zero time point ($p < 0.05$). There was no significant difference in the total activity recovered at each time (3642 ± 200 pmol/min/g, mean \pm S.E., $n = 24$). For further details, see "Experimental Procedures."

from the cytosolic to the particulate fraction was not altered by the initial load.

Fig. 3 shows the effect of the frequency of nontetanic stimuli on the translocation of protein kinase C activity from the cytosol to the particulate fraction. In all other experiments reported in this study (e.g. Figs. 1 and 2), the frequency of impulses (each 0.1 ms) was 100 Hz. A frequency of this magnitude is regarded as tetanic. Fig. 3 shows that the threshold for tetanic stimuli is between 10 and 20 Hz and that maximal translocation of protein kinase C occurred at a frequency that was subtetanic.

The experiments of Fig. 4 examine the relationship between the number of tetanic stimuli and the translocation of protein kinase C. It was found that only one tetanic stimulus of 200-ms duration at the start of the 30-min period was required to translocate the enzyme fully (Fig. 4A). Thus, changes in diacylglycerol and phosphatidic acid were also assessed, and as shown in Fig. 1B, again only one stimulus was required to give maximal increases in both substances.

Glucose transport as determined by the accumulation of 2-deoxyglucose 6-phosphate in the gastrocnemius-plantaris-soleus muscle group *in vivo* was assessed as a function of the

TABLE III

Effect of load applied to the muscle during electrical stimulation

Rats were anesthetized and the skin of the left hindlimb removed. The sciatic nerve was exposed and cut to allow positioning of the distal end in a suction electrode. The gastrocnemius-plantaris-soleus muscle group was exposed by careful removal of overlying tissue. The knee was secured by the tibioapatellar ligament and the Achilles tendon attached via a steel wire to a Harvard Bioscience isometric transducer. The initial tension on the muscle was varied as shown, and the muscle group was then stimulated by applying 200-ms trains of impulses (each impulse 0.1-ms duration) at a frequency of 100 Hz every 2 s. The voltage was set to attain full fiber recruitment. The muscle group was rapidly excised and processed as described under "Experimental Procedures" for the determination of protein kinase C activity. Values given are means \pm S.E. with the number of animals shown in parentheses.

Load applied to muscle	g	Protein kinase C activity		
		Cytosolic fraction	Particulate fraction	Total
Non-stimulated	500	1450 \pm 250	2070 \pm 200	3250 \pm 450 (9)
Stimulated	0	328 \pm 100	2941 \pm 260*	3269 \pm 360 (3)
Stimulated	200	362 \pm 76	3110 \pm 65*	3472 \pm 141 (3)
Stimulated	500	455 \pm 90	2843 \pm 233*	3293 \pm 257 (9)
Stimulated	700	361 \pm 112	3122 \pm 194*	3483 \pm 306 (3)

* All stimulated values were significantly greater than nonstimulated values ($p < 0.01$) but were not significantly different from each other.

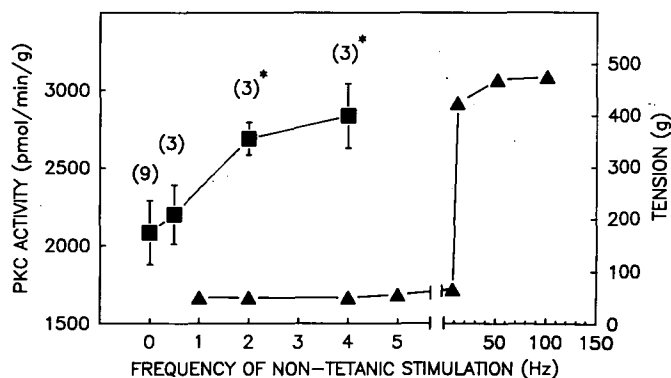


FIG. 3. Effect of frequency of nontetanic stimuli on tension development and on the translocation of protein kinase C (PKC) in rat muscle *in vivo*. Rats were anesthetized and the skin of the left hindlimb removed. The sciatic nerve was exposed and cut to allow positioning of the distal end in a suction electrode. The gastrocnemius-plantaris-soleus muscle group was then exposed, the knee secured, and the Achilles tendon attached to an isometric force transducer. The sciatic nerve was then stimulated by applying 200-ms trains of impulses (each impulse 0.1-ms duration) at variable frequency every 2 s for 2 min. The voltage was set to attain full fiber recruitment, and twitch tension (\blacktriangle) was recorded. The muscle group was then rapidly excised and processed as described under "Experimental Procedures" for the determination of protein kinase C activity in the particulate fraction. Each value represents the mean \pm S.E. of determinations done in duplicate with the number of animals shown in parentheses. *, Significantly different from unstimulated muscles ($p < 0.05$). There was no significant difference in the total activity recovered at each stimulation (3358 ± 215 pmol/min/g, mean \pm S.E., $n = 18$). For further details, see "Experimental Procedures."

number of tetanic stimuli. Fig. 5 shows that a minimum of 35 stimuli was required to activate glucose transport and that the maximal rate of uptake was still not reached even at 900 stimuli/30 min.

One of the disadvantages of the *in vivo* method for determining glucose uptake is that time courses cannot be conducted with precision. Thus, to determine the time course for electrical stimulation-activation of glucose transport, the iso-

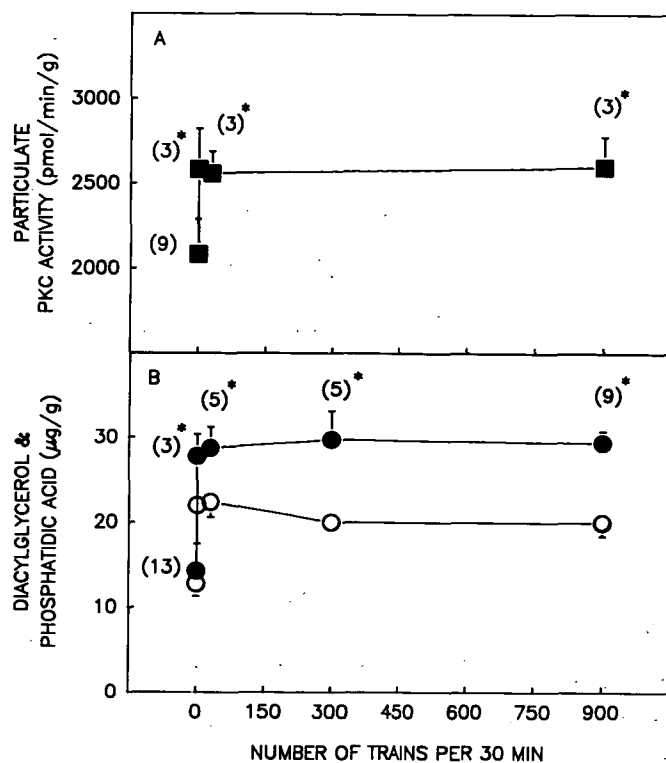


FIG. 4. Effect of the number of tetanic stimuli on the translocation of protein kinase C (PKC) and formation of diacylglycerol and phosphatidic acid. Rats were anesthetized and the skin of the left hindlimb removed. The sciatic nerve was exposed and cut to allow positioning of the distal end in a suction electrode. The gastrocnemius-plantaris-soleus muscle group was exposed, the knee secured, and the Achilles tendon attached to an isometric force transducer. The sciatic nerve was then stimulated by applying 200-ms trains of impulses (each impulse 0.1-ms duration) at 100 Hz. The voltage was set to attain full fiber recruitment. The total number of trains of tetanic stimuli initiated was as shown. After 30 min, the muscle group was excised and processed as described under "Experimental Procedures" for the determination of protein kinase C activity in the particulate fraction (A) and for the determination of diacylglycerol (\bullet) and phosphatidic acid (\circ) (B). Each value represents the mean \pm S.E. of determinations done in duplicate with the number of animals shown in parentheses. *, Significantly different from unstimulated muscles ($p < 0.05$). There were no significant differences in total activity recovered at each stimulation (3287 ± 205 pmol/min/g, mean \pm S.E., $n = 35$). For further details, see "Experimental Procedures."

lated soleus muscle was used. Despite differences in experimental conditions between the *in vivo* and the soleus *in vitro* systems, it was apparent that the activation of glucose transport by electrical stimulation was relatively slow to develop and required at least 20 min to reach maximum values (Fig. 6).

DISCUSSION

These results demonstrate for the first time that electrical stimulation of the sciatic nerve leads to changes consistent with protein kinase activation in the gastrocnemius-plantaris-soleus muscle group of rat hindlimb. A principal finding was the time-dependent translocation of the enzyme from the cytosol to the particulate fraction accompanied by a 2-fold increase in the concentration of diacylglycerol and phosphatidic acid. The association between the change in concentration of diacylglycerol and the translocation appeared to be causal as the increase in diacylglycerol (i) preceded the translocation of protein kinase C; (ii) remained elevated when stimulation of the sciatic nerve was halted and thus could be

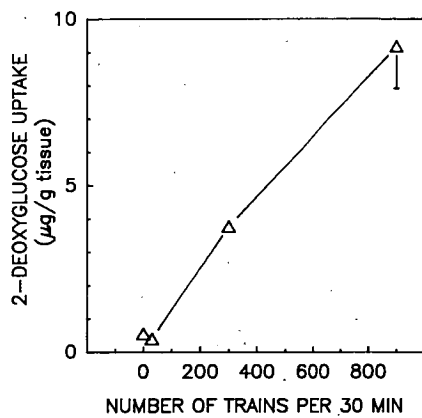


FIG. 5. Effect of the number of tetanic stimuli on the uptake of 2-deoxyglucose by the gastrocnemius-plantaris-soleus muscle group *in vivo*. Rats were anesthetized, the carotid artery was cannulated, and the skin of the left hindlimb removed. The sciatic nerve was exposed and cut to allow positioning of the distal end in a suction electrode. The gastrocnemius-plantaris-soleus muscle group was exposed, the knee secured, and the Achilles tendon attached to an isometric force transducer. 2-Deoxy[1- 3 H]glucose (30 μ Ci) was infused via the carotid artery, and samples of blood were removed every 5 min for the determination of the concentration of glucose and 2-deoxyglucose (cpm). Electrical stimulation of the sciatic nerve commenced immediately following the injection of labeled 2-deoxyglucose. Each train of electrical stimuli (tetanic stimuli) of 200-ms duration comprised 20 impulses of 0.1-ms duration at 10–20 V. Multiple trains were spaced 2 s apart. The gastrocnemius-plantaris-soleus muscle group was exercised at steady state (30 min) for the determination of 2-deoxyglucose 6-phosphate concentration. Values shown are means \pm S.E. for a minimum of three animals at each point. For further details, see "Experimental Procedures."

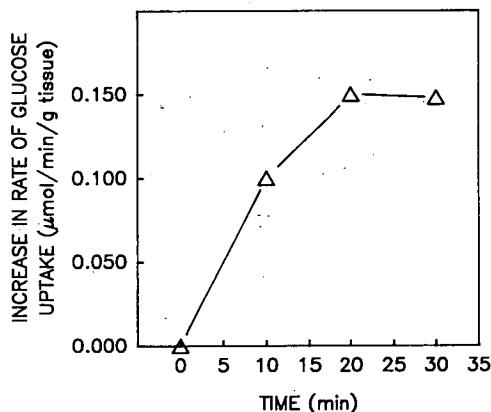


FIG. 6. Time course of contraction-induced increase in glucose transport by isolated soleus muscles. Muscles were mounted by the tendons on a muscle suspension apparatus (18) and incubated in medium containing 50 mM 3-O-methyl[1- 3 H]glucose (0.4 μ Ci/ml) and 5 mM [U- 14 C]sucrose (0.125 μ Ci/ml). One end of the muscle was attached to the electrode, which doubled as a gas inlet (18), and the other was attached via a steel wire to a Harvard Bioscience Isometric Transducer. Tension development was recorded during electrical stimulation (200-ms trains of impulses, each of 1-ms duration at a frequency of 100 Hz every 2 s). The voltage was adjusted (10–20 V) to attain full fiber recruitment. Muscles were then removed, rinsed in fresh buffer, blotted dry, and homogenized for the determination of radioactivity. Results shown are mean \pm S.E. from six muscles at each time point (error bars are within the symbols) and are expressed in terms of the increase in the rate of uptake where the basal rate was 0.13 ± 0.02 μ mol/min/g. For further details, see "Experimental Procedures."

the cause of a sustained translocation of protein kinase C; and (iii) occurred as the result of a single tetanic stimulus, the last also being sufficient to translocate the enzyme fully.

Diacylglycerol is regarded as a key regulator protein kinase C (2). Its production from membrane phospholipids is thought to be the trigger for protein kinase C activation by initiating a translocation from the cytosol to the membrane in which other activators of the enzyme such as phosphatidylserine are present. Thus, providing Ca^{2+} is also present, a quaternary complex consisting of Ca^{2+} , phospholipid, diacylglycerol, and the kinase itself (23) leads to activation of protein kinase C. In the present study, the origin of the increased concentration of diacylglycerol is not clear. The measurements of diacylglycerol present *net* accumulation and do not permit an assessment of the effects of muscle contraction on diacylglycerol formation and breakdown. The fatty acid analyses indicate that inositol phospholipids are probably precursors to the diacylglycerol pools in skeletal muscle because of the relatively high content of arachidonic acid. However, the high content of palmitate may suggest that triacylglycerol hydrolysis is also a contributor, although the total absence of oleate in the diacylglycerol weakens this argument. The origin of the phosphatidic acid is also unclear. Explanations must be consistent with observations that the increase in concentration of this substance closely parallels that of diacylglycerol (Fig. 1C). The fatty acid composition of the newly formed phosphatidic acid was similar to that of the diacylglycerol and was therefore suggestive that both substances were hydrolysis products of the same phospholipid. If this is the case, then diacylglycerol and phosphatidic acid may be formed simultaneously by the actions phospholipases C and D, respectively, or phospholipase C and diacylglycerol kinase, respectively. Other workers have already reported agonist-mediated rapid formation of phosphatidic acid in other systems (24). There is also some evidence that phosphatidic acid can substitute for diacylglycerol in the activation of protein kinase C (25).

Perhaps an unexpected finding was that the concentrations of both diacylglycerol and phosphatidic acid remained elevated even after resting the muscle for periods up to 30 min poststimulation. This is surprising, as in other systems metabolism of diacylglycerol and phosphatidic acid appears to be rapid (e.g. Refs. 26 and 27). Such an occurrence would imply that stimulation leads to a prolonged effect on the lipid-metabolizing enzymes responsible for diacylglycerol and phosphatidic acid production.

An important observation from the present study was that maximal translocation of protein kinase C occurred at sub-tetanic frequencies. This finding suggested that protein kinase C activation is an event closely dependent on membrane depolarization rather than a response following full tension development.

The present study also examines the relationship among muscle contraction, protein kinase C activation, and changes in the rate of muscle glucose transport. This was considered important because the mechanism by which contractile activity induces severalfold increases in the transport rate of glucose into muscle fibers is still not understood. Indeed, until recently, a permissive amount of insulin was considered essential (28). This now appears not to be the case, and the effects of exercise and insulin on muscle glucose transport are independent (29). Energy state, increased blood flow, hypoxia, and other factors have been considered (30) but largely dismissed as key events in the exercise-mediated increase in glucose uptake by muscle. The rise in the cytoplasmic Ca^{2+} level induced by muscle excitation would appear to be a logical candidate and has been suggested by others (31, 32). There is some evidence to suggest that excitation-contraction coupling in muscle and the resultant mobilization of intracellular Ca^{2+} may involve inositol phosphates (3–6) produced by rapid

hydrolysis of phosphatidylinositol 4,5-bisphosphate. Thus, a rise in intracellular Ca^{2+} and the production of diacylglycerol would occur concomitantly, and activation of protein kinase C could be expected. However, a drawback of the notion that Ca^{2+} is involved in the control of muscle glucose transport is the finding that Ca^{2+} concentrations return to basal levels immediately after each contraction ceases even though glucose transport remains elevated for a much longer period (33).

Comparisons between the actions of insulin and muscle contraction are useful as both act to increase muscle glucose transport, and mechanisms may be shared at least to some extent. The suggestion that protein kinase C may be important in some effects of insulin has been based in part on reports that insulin increases diacylglycerol levels (34–39) and activates protein kinase C (37). There is also much evidence that phorbol esters have insulin-like effects (34, 40–47), although it is clear that they do not completely mimic the actions of insulin (35, 37, 43, 45, 46, 48). Down-regulation of protein kinase C by phorbol esters in muscle (46) with the retention of full response to insulin has been argued as evidence against protein kinase C involvement in the action of this hormone. However, the link between protein kinase C activation and glucose transport stimulation is substantial. It includes observations that the glucose transporter protein from human red blood cells is a substrate for protein kinase C (7) and that activation of protein kinase C by phorbol esters has been observed in association with increased glucose transport as well as phosphorylation of the glucose transporter (8).

In the present study, it was noted that increases in the levels of diacylglycerol and phosphatidic acid preceded protein kinase C translocation *in vivo*. Time-dependent changes in glucose transport rates could not be determined *in vivo*. However, time course studies of the activation of this process in the isolated soleus muscle suggested that it was slower to develop than protein kinase C translocation. Thus, a causal relationship between electrical stimulation and the increase in glucose transport involving protein kinase C activation may exist. Even so, the data of Figs. 4 and 5 appear to be less supportive of that view as activation of protein kinase C required far fewer tetanic stimuli than did glucose transport. As alluded to above, it is unclear as to whether protein kinase C translocation leads necessarily to activation. Protein kinase C is a Ca^{2+} -dependent enzyme. During each muscle contraction, there is a transient increase in the intracellular level of Ca^{2+} . As the period of stimulation increases, so does the exposure to elevated Ca^{2+} levels, and the enzyme has sufficient time to cause a significant amount of catalysis.

As noted throughout this study, the extent of protein kinase C redistribution with muscle excitation was relatively small (20% change). Even though the enzyme appeared to co-purify with skeletal muscle sarcolemma (see "Results"), it is possible that the observed effect may be due to a relatively larger effect on only a portion of the cellular population. Further studies will be required to determine whether protein kinase C translocation is occurring in the same cells as those in which excitation is acting to increase glucose transport and whether it is involved in the control of the latter. To this end we have noted recently that phorbol ester-mediated down-regulation of protein kinase C in isolated soleus muscles led to a loss of contraction-induced increase in glucose transport without loss of glycogenolytic and contractile responses.⁴

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Long-term treatment of isolated rat soleus muscle with phorbol ester leads to loss of contraction-induced glucose transport

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Muscle contraction involves mobilization of intracellular Ca^{2+} and is associated with several metabolic adjustments, including increased glucose transport. In the present study isolated rat soleus muscles were exposed to 12-*O*-tetradecanoylphorbol 13-acetate, and responses to both insulin and contraction in terms of glucose transport were assessed. Muscles treated with this phorbol ester for 12 h showed an increased basal rate of 3-*O*-methylglucose uptake, and responded partially to insulin but did not respond to contraction. Phorbol-ester-treated and non-treated (vehicle-only) muscles were indistinguishable in terms of pre-contraction content of adenine nucleotide, phosphocreatine, lactate and glycogen, as well as contractile performance and contraction-induced glycogenolysis. Phorbol ester treatment of isolated solei for 12 h resulted in the loss of 90 % of protein kinase C activity as determined with histone IIIs as substrate, and 70 % as determined by using phorbol ester binding. It is concluded that treatment of solei with phorbol ester gives rise to a marked loss of contraction-induced glucose transport.

INTRODUCTION

Contraction of skeletal muscle results in an increase in glucose transport (for review see [1]), and until recently this increase was considered to require the presence of a 'permissive' amount of insulin [2]. Such a relationship implies that muscle contraction leads to a markedly increased sensitivity to the prevailing insulin concentration. This now appears not to be the case, and the effects of exercise and insulin on muscle glucose transport appear to be independent [3,4]. However, the mechanism by which contraction stimulates glucose entry into muscle remains to be described [1], although an increase in glucose transporters in the plasma membrane now appears likely [5,6].

Comparisons between the actions of insulin and muscle contraction have been made [1], as both act to increase muscle glucose transport and mechanisms may be shared at least to some extent. Protein kinase C (PKC), a Ca^{2+} - and phospholipid-dependent serine/threonine kinase [7] may be important in some effects of insulin. Experiments have been reported where insulin increases diacylglycerol levels [8–13] and activates PKC [11]. There is also considerable evidence that phorbol esters such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) have insulin-like effects [14–21], although it is clear that they do not completely mimic the actions of insulin [9,11,17,19,20,22]. Activation of PKC has also been suggested to be involved in modulating responses to insulin [17,21,23–25]. Exposure of intact cells to TPA results in serine phosphorylation of insulin receptors, and incubation of isolated insulin receptors with purified PKC is associated with a decrease in the insulin receptor's basal and insulin-stimulated tyrosine kinase activity [25,26]. In addition, exposure to TPA results in a decreased affinity of the insulin receptor for insulin [17,25].

Attempted down-regulation of PKC by phorbol esters in muscle has led to mixed results. Klip & Ramlal [20] found that treatment of a cultured muscle cell line with TPA resulted in the down-regulation of PKC as assayed by phosphorylation of lysine-rich histone (histone IIIs), but with the retention of full

response to insulin. In another muscle cell line Cooper *et al.* [27] reported that TPA treatment resulted in loss of PKC-directed histone phosphorylation and loss of TPA effects on glucose transport, but PKC-dependent vinculin phosphorylation by type II isoenzyme was retained. This latter observation appeared to explain the continued effectiveness of both insulin and diacylglycerol for stimulating glucose transport in the 'down-regulated' cells [27]. In a third study, involving isolated mouse soleus muscles, Tanti *et al.* [28] reported that down-regulation of PKC, as assayed with lysine-rich histone, inhibited the responses to both TPA and insulin.

Recently we have been exploring the relationship between contraction-induced glucose uptake in skeletal muscle and the involvement of PKC in the control of this process [29]. Our findings indicate that electrical stimulation of skeletal muscle initiates a rapid formation of diacylglycerol and phosphatidate as well as the translocation of PKC, and that these changes precede the increase in glucose uptake [29]. In the present study isolated soleus muscles were incubated with TPA for 12 h and the response of the muscles to electrical stimulation, as well as to insulin, was assessed. PKC activity was monitored by using lysine-rich histone as substrate as well as phorbol ester binding.

MATERIALS AND METHODS

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was purchased from Bresatec (Adelaide, Australia). 3-*O*-methyl[1- ^3H]glucose (^3H]3-OMG), [20(n)- ^3H]phorbol 12,13-dibutyrate (^3H]PDBu) and [U- ^{14}C]sucrose were obtained from Amersham (Bucks., U.K.). Histone IIIs, diolein, phosphatidylserine, TPA, PDD (4 α -phorbol 12,13-didecanoate), as well as coenzymes, enzymes and substrates were obtained from Sigma (St. Louis, MO, U.S.A.). DE-52 DEAE-cellulose and P81 phosphocellulose paper were purchased from Whatman (Maidstone, U.K.). Insulin (Actrapid MC) was from Novo Industri A/S (Copenhagen, Denmark).

Female Hooded Wistar rats (55–65 g) were anaesthetized with

Abbreviations used: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; ^3H]3-OMG; 3-*O*-methyl[1- ^3H]glucose; DMSO, dimethyl sulfoxide; ^3H]PDBu, [20(n)- ^3H]phorbol 12,13-dibutyrate; PDD, 4 α -phorbol 12,13-didecanoate.

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pentobarbital (5 mg) intraperitoneally. The soleus muscles were removed and attached via the tendons to stainless-steel U-shaped supports (15 mm in width) designed to maintain a tension of approx. 10 g. Muscles were incubated with shaking (100 oscillations/min) in pairs at 30 °C in stoppered 50 ml flasks which contained 5 ml of incubation medium. The incubation medium consisted of modified Krebs–Ringer saline/bicarbonate buffer (93 mM-NaCl, 4.7 mM-KCl, 1.2 mM-KH₂PO₄, 1.2 mM-MgSO₄, 0.05 mM-Na₂EDTA, 1.27 mM-CaCl₂, 5 mM-glucose and 25 mM-NaHCO₃) gassed with O₂/CO₂ (19:1) to give pH 7.4 and contained 0.1% freeze-dried serum obtained from healthy rats. The total glucose concentration was adjusted to 5 mM; other additions as indicated were 35 mM-mannitol, 100 μ units of insulin/ml, PDD [in dimethyl sulphoxide (DMSO)], and TPA (in DMSO) or DMSO. The flasks were continuously gassed with O₂/CO₂ (19:1) and the medium was changed every 4 h. Muscles were incubated for various times, then removed from the flasks and washed in 25 ml of insulin-free medium before assessment of glucose-uptake rate, metabolite contents, PKC activity and phorbol ester binding.

For determination of glucose uptake, solei were re-mounted by the tendons in a muscle-suspension apparatus, similar to that described by Nesher *et al.* [30]. The incubation medium (4 ml) was modified Krebs–Ringer bicarbonate buffer of the following composition: 93 mM-NaCl, 5 mM-KCl, 1.2 mM-KH₂PO₄, 1.2 mM-MgSO₄, 0.05 mM-Na₂EDTA, 1.27 mM-CaCl₂, 25 mM-NaHCO₃, 5 mM-Hepes, 5 mM-pyruvate, 50 mM-[³H]3-OMG (10 μ Ci/ml) and 5 mM-[U-¹⁴C]sucrose (3.2 μ Ci/ml). The medium was gassed with O₂/CO₂ (19:1) and the pH was adjusted to 7.4. Gas flow was broken into small bubbles to provide adequate mixing of the incubation media. Two muscles were mounted simultaneously, and details for electrical stimulation (Hugo Sachs Elektronik Nerve Muscle Stimulator Type 214) were as follows: 200 ms train of impulses (each impulse 1 ms duration) at a frequency of 50 Hz every 2 s, and the voltage was adjusted (10–20 V) to attain full fibre recruitment. Tension development was recorded with a Harvard Bioscience Isometric Transducer. Solei were frozen (liquid N₂) and then homogenized in 2 ml of water (Ultra-Turrax, 2 \times 30 s), centrifuged (15 min, 220 g), and 1.5 ml of the supernatant was added to 12 ml of ASC II (Amersham) for radioactivity counting. Inclusion of [U-¹⁴C]sucrose in the incubations enabled correction for the extracellular space.

ATP, ADP, AMP, phosphocreatine and lactate were determined in neutralized perchlorate extracts of frozen muscle. The adenine nucleotides were determined by h.p.l.c. (Waters) with a Pharmacia Mono Q anion-exchange column. AMP, ADP and ATP were eluted in order with a linear gradient of 4.5–150 mM-(NH₄)₂SO₄ in 20 mM-Tris/HCl, pH 8.0, and detected at 254 nm. Phosphocreatine, lactate and glycogen were determined by standard methods [31].

For PKC activity determinations, four solei were homogenized in 4 ml of ice-cold 20 mM-Tris/HCl buffer, pH 7.5, containing 250 mM-sucrose and 1 mM-dithiothreitol, with an Ultra-Turrax homogenizer (2 \times 30 s). The homogenate was centrifuged (1 h, 100 000 g) and the resulting pellet (particulate fraction) extracted (1 h) with 6 ml of 20 mM-Tris/HCl buffer, pH 7.5, containing 2.0% (v/v) Triton X-100, 2 mM-EDTA, 10 mM-EGTA and 1 mM-dithiothreitol. The supernatant was collected by centrifugation (30 min, 100 000 g).

Supernatant extracts containing PKC were applied to columns (4 ml) of Whatman DE-52, which were then washed with 20 ml of 20 mM-Tris/HCl, pH 7.5, containing 2 mM-EDTA, 0.5 mM-EGTA and 1 mM-dithiothreitol. PKC activity was eluted with a linear gradient (36 ml) of 0–0.15 M-NaCl [32]; 4 ml fractions were collected, and typically PKC activity was found in fractions 4–6, corresponding to 30–90 mM-NaCl. Fractions were assayed

for PKC [33] by using histone III_s as the substrate and Whatman phosphocellulose P81 paper to collect the acid-precipitable material. Activity was calculated from the difference in ³²P incorporated into histone in the presence and absence of added phospholipids/Ca²⁺ and was expressed as pmol of ³²P incorporated/min per g fresh wt. of muscle.

Binding of [³H]PDBu was determined by the filtration method [34]. Muscle homogenates were processed as above for the partial purification of PKC. Fractions from the DE-52 column containing PKC activity were pooled from the particulate material and concentrated to approx. 1 ml in an Amicon concentrating system (YM30 membrane). Phorbol-ester-binding reaction mixtures contained 25 mM-Tris/HCl, pH 7.5, 10 mM-magnesium acetate, 1.4 mM-CaCl₂, 0.4 mM-EGTA, 0.4 mM-EDTA, 4 mg of BSA/ml, 100 μ g of phosphatidylserine/ml, 20 nM-[³H]PDBu, with or without 3 μ M-TPA, in a total volume of 400 μ l in glass tubes (10 mm \times 75 mm). After addition of 400 μ l of concentrated sample, the tubes were incubated overnight at 4 °C. Bound [³H]PDBu was separated from free [³H]PDBu by adding 1 ml of 20 mM-Tris/HCl (pH 7.5)/10 mM-magnesium acetate/1 mM-CaCl₂ and filtering the mixture through 2.4 cm-diam. Whatman GF/C filters by suction. The tubes and filters were washed with 5 \times 1 ml of filtering solution, dried in air and then counted for radioactivity. Specific binding was calculated as total binding minus non-specific binding observed in the presence of 3 μ M-TPA.

Data were evaluated with Student's *t* test, and *P* < 0.05 was taken as being statistically significant.

RESULTS

Effect of long-term treatment with TPA on indices of viability

Extended incubation of isolated solei may lead to loss of viability, and agents such as those used in the present study (TPA and DMSO) could exacerbate this effect. Important considerations to minimize loss of function included muscle size [35,36], incubation temperature and medium composition. Several of these were varied, and the following indices of viability determined: water content, ATP, ADP, AMP, phosphocreatine, lactate and glycogen. Indices of incubated muscles were compared with those of freshly excised solei. Components of the incubation medium found to be protective were rat serum, mannitol, insulin and glucose. It was also noted that muscles were best protected when incubated at 30 °C (results not shown). Table 1 compares freshly excised solei with muscles incubated for 12 h at two concentrations of TPA and two concentrations of vehicle. Even the highest concentration of TPA used (5 μ M) did not significantly affect any of the metabolites or the water content of isolated solei (Table 1).

Effect of long-term treatment with TPA on insulin- and contraction-induced transport of 3-OMG

Table 2 shows that freshly isolated solei responded to insulin and electrical stimuli by increasing the rate of 3-*O*-MG uptake. For insulin (10 munits/ml), the rate increased by 70%, from 160 to 272 nmol/min per g, and for electrical stimulation the rate increased by 89%, to 302 nmol/min per g. The magnitude of these responses was less than noted for soleus muscle in perfused hindlimb [37] or *in vivo* [38], but was similar to studies *in vitro* [28,39].

Muscles incubated for 12 h with no additions, 0.06% DMSO or 0.3% DMSO each responded to insulin and electrical stimulation to the same extent as freshly excised solei. Treatment with TPA resulted in a partial loss of response to insulin. For 1 μ M-TPA the insulin response decreased from 61 to 40%, and for 5 μ M from 57 to 24%. At the higher concentration of TPA the

Table 1. Adenine nucleotides, phosphocreatine, lactate and glycogen contents of isolated solei

Solei either were freshly excised* or had been incubated at 30 °C for 12 h in buffered serum containing TPA in DMSO, or DMSO alone. Muscles were then removed for determination of water content (by drying at 110 °C for 15 h) and metabolites. Glycogen is expressed as μmol of glucose. Means \pm S.E.M. are shown for three observations.

Treatment	Water content ($\mu\text{l/g}$ wet wt.)	Metabolite concn. ($\mu\text{mol/g}$ dry wt.)					
		ATP	ADP	AMP	Phosphocreatine	Lactate	Glycogen
None*	826.4 \pm 8.0	21.6 \pm 1.3	4.1 \pm 1.1	1.0 \pm 0.1	39.2 \pm 2.0	11.5 \pm 0.6	98.1 \pm 21.1
0.06 % DMSO	853.6 \pm 8.1	23.6 \pm 3.5	3.1 \pm 0.5	1.9 \pm 0.1	40.6 \pm 2.0	16.0 \pm 2.2	82.6 \pm 7.2
1 μM -TPA	840.2 \pm 11.8	18.1 \pm 0.8	2.4 \pm 0.2	1.2 \pm 0.3	41.0 \pm 3.8	13.2 \pm 2.5	84.7 \pm 4
0.3 % DMSO	788.1 \pm 17.3	21.7 \pm 1.1	3.3 \pm 0.4	1.1 \pm 0.1	38.7 \pm 1.4	13.18 \pm 1.7	90.5 \pm 8.6
5 μM -TPA	821.4 \pm 8.5	20.6 \pm 2.4	3.3 \pm 0.5	1.0 \pm 0.2	41.0 \pm 1.2	17.35 \pm 1.5	93.0 \pm 16.1

Table 2. Effect of pretreatment of solei with phorbol esters on insulin- and contraction-induced 3-OMG transport

Solei either were freshly excised† or had been incubated at 30 °C for 12 h in buffered serum containing TPA in DMSO, PDD in DMSO or DMSO alone. Muscles were then transferred to a muscle suspension apparatus containing [^3H]3-OMG and [$\text{U-}^{14}\text{C}$]sucrose for an additional 30 min. Uptake of [^3H]3-OMG was determined at 37 °C as described in the text, and means \pm S.E.M. are shown with the numbers of observations given in parentheses: * $P < 0.05$, [^3H]3-OMG uptake was greater than corresponding 'no addition' control; n.s., not significantly different from corresponding 'no addition' control.

Pretreatment	[^3H]3-OMG uptake (nmol/min per g)		
	No addition	Insulin (10 munits/ml)	Contractions (30 min)
None†	160 \pm 6 (3)	272 \pm 23 (4)*	302 \pm 14 (3)*
Incubated for 12 h	189 \pm 11 (4)	303 \pm 29 (4)*	283 \pm 22 (3)*
0.06 % DMSO	187 \pm 14 (4)	301 \pm 19 (4)*	300 \pm 22 (4)*
1 μM -TPA in 0.06 % DMSO	198 \pm 10 (5)	278 \pm 7 (5)*	259 \pm 15 (5)*
0.3 % DMSO	185 \pm 17 (5)	291 \pm 16 (5)*	288 \pm 13 (5)*
5 μM -TPA in 0.3 % DMSO	225 \pm 6 (5)	279 \pm 8 (5)*	211 \pm 10 (5) n.s.
5 μM -PDD in 0.3 % DMSO			297 \pm 17 (4)*

apparent loss of insulin response may be exaggerated, owing to the increased basal uptake rate. As shown in Table 2, the basal uptake rate increased from 189 \pm 11 to 198 \pm 10 (1 μM -TPA) and 225 \pm 6 (5 μM -TPA):

Treatment with 5 μM -TPA for 12 h markedly inhibited the response to electrical stimuli (Table 2). At 1 μM -TPA the response decreased from 60 % to 31 % and at 5 μM -TPA from 56 % to zero, as compared with the corresponding DMSO controls. Treatment with 5 μM -PDD, a non-active phorbol ester, had no effect on contraction-induced 3-OMG uptake (Table 2).

Effect of long-term treatment with TPA on contraction-induced twitch tension and glycogenolysis

Since long-term exposure of solei to TPA could produce a variety of changes in muscle biochemistry, some of which could be non-specific, it was considered important to assess the effects on responses related to glucose uptake. Table 3 shows the effect of incubation for 12 h at 30 °C on the twitch tension developed by solei which were subsequently stimulated to contract. Incubation for 12 h without addition showed no loss of contractile performance. TPA had no significant effect as compared with the corresponding DMSO control (Table 3). Thus electrical stimulation of solei previously exposed to a concentration of TPA sufficient to lead to a total loss of glucose transport responded normally in terms of twitch tension development.

Table 1 shows that incubation of muscles for 12 h with or

Table 3. Effect of pretreatment of solei with TPA on electrical-stimulation-induced twitch tension

Solei either were freshly excised† or had been incubated at 30 °C for 12 h in buffered serum containing TPA in DMSO, or DMSO alone. Muscles were then transferred to a muscle suspension apparatus at 37 °C, and twitch tension was recorded during a 30 min period of electrical stimulation (200 ms train of 1 ms impulses at 50 Hz, every 2 s; the voltage was adjusted to attain full fibre recruitment). Means \pm S.E.M. are shown with the numbers of observations given in parentheses: n.s., not significantly different from corresponding DMSO control.

Pretreatment	Twitch tension (g)
None†	10.3 \pm 1.0 (5)
Incubated for 12 h	9.2 \pm 1.5 (4)
0.06 % DMSO	8.9 \pm 1.2 (7)
1 μM -TPA in 0.06 % DMSO	8.5 \pm 0.5 (5) n.s.
0.3 % DMSO	8.0 \pm 1.5 (3)
5 μM -TPA in 0.3 % DMSO	8.3 \pm 1.2 (4) n.s.

without TPA did not alter glycogen content, provided that glucose was present in the medium. If the muscles were then stimulated to contract for 30 min, the glycogen content decreased markedly from approx. 88 to 12.1 \pm 4.4 ($n = 3$) and 16.0 \pm 4.8

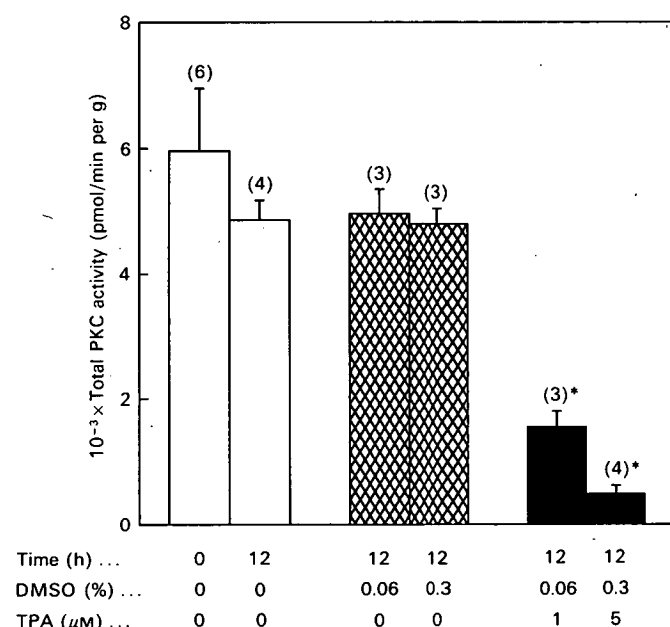


Fig. 1. Effect of pretreatment with TPA on total PKC activity

Solei attached to metal supports were incubated at 30 °C for 12 h in buffered serum containing TPA in DMSO or DMSO alone, as indicated. The muscles were then homogenized and the total activity (cytosolic+particulate) of PKC was determined. Means \pm S.E.M. (pmol of histone III_s phosphorylated/min per g wet wt. of muscle) are shown, with the numbers of observations in parentheses.

* $P < 0.01$, activity of TPA-treated muscles was less than corresponding DMSO control.

($n = 3$) $\mu\text{mol/g}$ dry wt. for 0.3 %-DMSO- and 5 μM -TPA-treated muscles, respectively. Thus loss of glucose transport was not associated with a loss of the glycogenolytic response to muscle contractions.

Effect of long-term treatment with TPA on PKC activity and phorbol ester binding

Long-term treatment of cells has been routinely used to 'down-regulate' PKC (e.g. see [40]). Thus in the present study muscles treated with 1 μM - and 5 μM -TPA and which showed loss of contraction-induced glucose transport were assayed for PKC activity. Two methods were used. The first was based on the conventional assay with lysine-rich histone (histone III_s) as substrate [33]. Fig. 1 shows that exposure of solei to 1 μM - and 5 μM -TPA for 12 h decreased total (cytosolic+particulate) activity of PKC by approx. 70 and 90 % respectively. In the second method total binding sites for [³H]PDBu were determined. Exposure of solei to 5 μM -TPA for 12 h resulted in a decrease in binding from 2.68 ± 1.11 ($n = 4$; 0.3 % DMSO) to 0.81 ± 0.28 ($n = 4$) pmol/g wet wt.

DISCUSSION

The key finding in the present study was that muscles exposed to TPA for 12 h responded comparably with appropriate controls in terms of tension development and glycogen breakdown, but failed to respond in terms of glucose transport. Since long-term exposure to TPA is now a well-accepted procedure for down-regulation of PKC, it appears likely that the accompanying loss of PKC from the solei was responsible for the loss of contraction-induced glucose transport. Indeed, inspection of the data of Table 2 and Fig. 1 show that down-regulation of PKC by 70 %

coincided with a loss of approx. 50 % of the contraction-induced response, and at higher doses of TPA, when PKC was down-regulated further (by 90 %), a total loss occurred. However, the conclusion that PKC is involved in the metabolic adjustments to muscle contraction, particularly glucose transport, must be treated with caution. There are now several identified isoenzymes of PKC [41] which have different substrate specificities and which may be differentially down-regulated by TPA treatment [18,27,42]. Thus TPA treatment of KM3 cells has been reported to cause translocation and depletion of type II PKC more quickly than for type III enzyme, although both sub-species were totally depleted in 2 h [42]. Perhaps more importantly, when vinculin was used as substrate for the determination of PKC activity [27], TPA treatment of BC3H-1 myocytes did not deplete type II enzyme, even though this sub-species was undetectable with histone in the same TPA-treated cells. Thus in the present study it is possible that the apparent down-regulation of PKC as determined by histone III_s kinase activity (Fig. 1) did not reflect down-regulation of all sub-species. To address this issue, we have made use of the observations by others that skeletal muscle contains predominantly types II and III PKC [43] and that these types bind [³H]PDBu with similar affinity [44]. Clearly, the marked loss of total [³H]PDBu binding which accompanied the 12 h treatment of solei with 5 μM -TPA would support the view that down-regulation of both types II and III has occurred.

A further consideration is that the loss of contraction-mediated glucose uptake results not from down-regulation of PKC but from the 12 h period of exposure to TPA as a PKC activator. Sowell *et al.* [45] have shown that treatment of solei with TPA leads to a marked inhibition of glycogen synthesis, and there is some indication in the present study and from others [20] that the basal rate of glucose transport is increased by prolonged TPA exposure. In addition, the observation that short-term exposure by phorbol esters induces symptoms of myotonia in skeletal muscle by lowering chloride conductance [46] suggests that ion channels may be modified by PKC activator. Together these changes may be representative of a general metabolic and functional adjustment to chronic PKC activation, which could include loss of contraction-induced glucose transport.

The partial loss of insulin response (Table 2) as a result of long-term treatment with TPA might also suggest that PKC is involved, at least in part, in the action of insulin. This would be consistent with a recent report by Tanti *et al.* [28], but with some difference. In their studies [28] it was reported that 24 h exposure of isolated mouse solei to 1 μM -TPA led to a 40 % loss of cytosolic PKC activity and a total loss of insulin-mediated 2-deoxyglucose uptake. However, differences between the species may be contributory. For example, 2-deoxyglucose uptake by mouse solei is stimulated by TPA [28]. For rat solei 2-deoxyglucose uptake is not affected by 0.04–1.6 μM -TPA [45]. Similarly, in our laboratory 3-OMG uptake by isolated rat solei was not stimulated by acute exposure to 1 μM -TPA [117 ± 10 ($n = 5$) versus 127 ± 21 nmol/min per g ($n = 7$) for control muscles] or 200 μM -1,2-diocanoyl-*rac*-glycerol [79 ± 2 ($n = 3$) versus 96 ± 16 nmol/min per g ($n = 3$) for matched control muscles].

Comparison of skeletal muscle with other tissues suggests that PKC activity is low (e.g. see ref. [7]) and may be rendered functionally inoperative by the accompanying presence of relatively large amounts of an endogenous inhibitor of PKC [47]. However, when assayed under comparable conditions, the levels of PKC and the cyclic AMP-dependent protein kinase (protein kinase A) are similar in muscle [7], and therefore PKC may have as great a significance to muscle metabolism as does protein kinase A.

Finally, the link between muscle contraction and glucose

transport is still far from clear. Even though the present and our previous studies [29] imply a role for PKC and generally support an association between phosphoinositol metabolism and excitation-contraction coupling [48,49] it will require further studies to establish that PKC is an activator of glucose transport. There have been two recent reports [5,6] indicating that acute exercise increased the number of plasma-membrane glucose transporters in rat skeletal muscle. This may result from translocation of glucose transporters from a microsomal fraction to the muscle sarcolemma, and therefore may be similar to insulin [50], or may result from changes in the intrinsic activity of the glucose transporter. However, it is likely that the mechanisms involved in insulin and exercise differ in some respect, as the effect of a maximal concentration of insulin on glucose transport was further increased by muscle contraction [51].

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A ROLE FOR PROTEIN KINASE C IN THE INSULIN-LIKE ACTION OF MUSCLE CONTRACTION ON GLUCOSE TRANSPORT?

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Until recently glucose uptake due to muscle contraction was considered to require the presence of a "permissive" amount of insulin (1). Such a relationship implied that muscle contraction led to a markedly increased sensitivity to the prevailing insulin concentration. This now appears not to be the case and the effects of exercise and insulin on muscle glucose transport appear to be quite independent (2) and the mechanism by which contraction stimulates glucose entry into muscle remains to be described. It has been argued by several research groups that information on the molecular mechanisms involved in contraction-induced glucose uptake may clarify some of the molecular mechanisms of insulin itself.

Since excitation-contraction of muscle is associated with mobilization of intracellular Ca^{2+} there has been interest in the possibility that inositol phosphates (3) and as well the Ca^{2+} -activated phospholipid-dependent protein kinase (protein kinase C) may be involved in the metabolic adjustment. Interest in the latter has been heightened by reports that the glucose transporter from human red-blood cells is a substrate for brain protein kinase C (4) and that activation of protein kinase C in 3T3L1 adipocytes by phorbol esters leads to increased glucose transport as well as phosphorylation of the glucose transporter (5).

The Ca^{2+} -activated and phospholipid-dependent protein kinase (protein kinase C) has been shown to have a widespread distribution in mammalian tissues including skeletal muscle. This protein kinase can be activated by a low concentration of Ca^{2+} in the presence of both phosphatidyl serine and diacylglycerol. In addition it has been proposed that the release of diacylglycerol through the signal-induced breakdown of phosphatidyl inositol may trigger the activation of protein kinase C *in vivo* (6) reflected by translocation of this enzyme from cytosol to the plasma membrane or particulate fraction.

Recently we have developed an assay for protein kinase C in skeletal muscle preparations (7) and examined the content of this enzyme in different muscle types as well as the effect of contraction on the activation of the enzyme *in vivo* reflected by translocation from the cytosol to the particulate fraction.

Comparison of the activities of protein kinase C in soleus and gastrocnemius white muscle indicated that the former had a greater total and a greater particulate activity. These differences in protein kinase C activities and apparent state of activation of the enzyme may relate to the metabolic and physiological differences between these two muscle types. Soleus muscle which is postural, is rich in slow oxidative red fibres and is rich in mitochondrial enzymes. The soleus muscle also has a high resting capacity for glucose uptake and metabolism and as well, shows a marked increase in glucose uptake during exercise (8).

Studies on the effect of contraction on the activation of protein kinase C *in vivo* were conducted in conjunction with measurements of glucose uptake (9); the experimental systems were identical so that changes in glucose uptake and protein kinase C and its regulators could be compared.

The gastrocnemius-plantaris-soleus muscle group was stimulated electrically in anaesthetized rats via the sciatic nerve for intervals up to 30 min (200 ms train of 100 Hz applied every 2 s) adjusted (10-20 volts) to attain full fibre recruitment. This muscle group was rapidly excised and homogenized for the determination of particulate and soluble protein kinase C activities (7) or for the determination of diacylglycerol (DG) and phosphatidic acid (PA) by thin layer chromatography/Coomassie Blue-laser densitometry. Electrical stimulation led to a time-dependent translocation of protein kinase C from the muscle cytosol to the particulate fraction and an increase in glucose transport. Maximum translocation occurred at 2 min and coincided with a two-fold increase in the concentration of both DG and PA. Time courses for DG and PA formation as well as for protein kinase C translocation were identical. Stimulation for periods of up to 30 min showed no loss of total protein kinase C activity. There was no reversal of protein kinase C translocation even after 30 min following a 5 min period of stimulation. Translocation of protein kinase C was not influenced by variations in applied load (0-750 g) at maximal fibre recruitment but was dependent on the number of stimuli/min. However translocation of protein kinase C was achieved at a frequency of stimuli which was considerably less than that required for the activation of glucose transport.

It is concluded that electrical stimulation of skeletal muscle activates either directly or indirectly enzyme(s) responsible for the production of diacylglycerol and that this may be responsible for the translocation and activation of protein kinase C. It remains to be established whether protein kinase C is involved in immediate metabolic or longer term (e.g. growth) adjustments to exercise but exercise-induced translocation (activation?) of protein kinase C does not appear to be involved in the activation of glucose transport.

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CONTRACTION-ASSOCIATED TRANSLOCATION OF PROTEIN KINASE C IN RAT SKELETAL MUSCLE

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Excitation-contraction coupling of skeletal muscle and the resultant mobilization of intracellular Ca^{2+} may involve inositol phosphates (1) produced by rapid hydrolysis of phosphatidyl inositol. Such findings imply that diacylglycerol is also produced and that protein kinase C (PKC) may be involved in metabolic changes induced by muscle stimulation. Thus in the present study we have developed an assay for PKC in skeletal muscle preparations and examined the effect of contraction on the activation of protein kinase C *in vivo* (2) reflected by translocation of this enzyme from cytosol to the plasma membrane or particulate fractions. The gastrocnemius-plantaris-soleus muscle group was stimulated electrically in anaesthetized rats via the sciatic nerve for 0, 1, 2 or 5 min (200 ms train of 100 Hz applied every 2 s) adjusted (10-20 volts) to attain total fibre recruitment. This muscle group was rapidly excised and homogenized and the cytosol and particulate fractions were prepared by centrifugation. PKC activity was resolved by DE-52 anion exchange chromatography of a 2% Triton X-100/10 mM ECTA extract of the particulate fraction and a desalted $(\text{NH}_4)_2\text{SO}_4$ cut (21-45%) of the soluble fraction; both forms eluting at 50 mM NaCl. Recovery of total activity by this method was >95% and the enzyme was separated from other interfering enzymes and endogenous inhibitors. Electrical stimulation led to a time-dependent translocation of protein kinase C from the muscle cytosol to the particulate fraction. Maximum translocation occurred at 2 min then declined over the next 3 min. The loss of activity from the cytosol was accounted for entirely by the increase in activity in the particulate fraction. There was no evidence that translocation resulted from the contraction-induced increase in muscle blood content. Translocation of PKC may imply a role for this enzyme in contraction-initiated changes in muscle metabolism including glucose transport (3).

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(Cell Calcium Metabolism '87: p. 231. U.S.A.)

EXERCISE-INDUCED TRANSLOCATION OF PROTEIN KINASE C AND PRODUCTION OF DIACYLGLYCEROL AND PHOSPHATIDIC ACID IN RAT SKELETAL MUSCLE *in vivo*

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Excitation-contraction coupling of skeletal muscle and the resultant mobilization of intracellular Ca^{2+} may involve inositol phosphates (1) produced by rapid hydrolysis of phosphatidyl inositol phosphate. In addition PKC may be activated by the production of diacylglycerol. Thus in the present study we have developed an assay for protein kinase C (PKC) in skeletal muscle preparations and examined the effect of contraction on the activation of this enzyme *in vivo* reflected by translocation from the cytosol to the particulate fraction. The gastrocnemius-plantaris-soleus muscle group was stimulated electrically in anaesthetized rats via the sciatic nerve for intervals up to 30 min (200 ms train of 100 Hz applied every 2 s) adjusted (10-20 volts) to attain total fibre recruitment. This muscle group was rapidly excised and homogenized for the determination of particulate and soluble PKC activities (2) or for the determination of diacylglycerol (DG) and phosphatidic acid (PA) by thin layer chromatography/Coomassie Blue-laser densitometry. Electrical stimulation led to a time-dependent translocation of PKC from the muscle cytosol to the particulate fraction. Maximum translocation occurred at 2 min and coincided with a two-fold increase in the concentration of both DG and PA. Time courses for DG and PA formation as well as for PKC translocation were identical. Stimulation for periods of up to 30 min showed no loss of total PKC activity. There was no reversal of PKC translocation even after 30 min following a 5 min period of stimulation. Translocation of PKC was not influenced by variations in applied load (0-750 g) at maximal fibre recruitment but was dependent on the number of stimuli/min.

It is concluded that electrical stimulation of skeletal muscle activates either directly or indirectly enzyme(s) responsible for the production of diacylglycerol and that this is responsible for the translocation and activation of PKC. It remains to be established whether PKC is involved in immediate metabolic or longer term (e.g. growth) adjustments to exercise.

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(Can. J. Sport Sci. 13:p.9p., 1988)

SP 14

DOWN-REGULATION OF PROTEIN KINASE C IN ISOLATED RAT SOLEUS MUSCLE RESULTS IN THE LOSS OF CONTRACTION-INDUCED GLUCOSE TRANSPORT

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Recent studies with the rat gastrocnemius-plantaris-soleus skeletal muscle group, *in vivo*, have shown a contraction-induced, time-dependent increase in the concentration of diacylglycerol and a corresponding translocation of PKC (1). Even though time dependent changes in glucose transport rate could not be determined *in vivo*, time-course studies of the activation of glucose transport in the isolated soleus muscle suggested that it was slower to develop than PKC translocation. Thus a causal relationship between electrical stimulation and the increase in glucose transport involving PKC activation may exist.

In this study the approach chosen was to down-regulate PKC with prolonged treatment of isolated soleus muscle with phorbol esters (TPA), and thereby directly assess the role of PKC in both insulin- and contraction-mediated glucose transport. Solei were removed from anaesthetized rats and attached via the tendons to stainless steel U-shaped supports designed to maintain a tension of approx. 10g. Muscles were incubated in 5 ml of modified Krebs-Ringer saline-bicarbonate buffer containing 0.1% rat serum, glucose (5mM), mannitol (35mM), insulin (100uU/ml) and TPA or DMSO and the medium was changed every 4 hr. For 3-O-methylglucose determination, the muscles were remounted in a special suspension apparatus which contained 4ml of modified Krebs-Ringer bicarbonate buffer and 3-O-methyl-[1-³H]glucose. Muscles were stimulated electrically for varying times (200ms train of impulses (each impulse of 1 ms duration) at a frequency of 50 Hz every 2 sec) adjusted (10-20 volts) to attain full fibre recruitment. Solei were either frozen (liquid N₂), until assayed for 3-O-methylglucose, ATP, ADP, AMP, creatine phosphate, lactate and glycogen, or homogenized and the soluble and particulate fractions were prepared by centrifugation. PKC was resolved by anion exchange chromatography of the soluble fraction and of a Triton X-100 extract of the particulate fraction.

TPA treatment of isolated solei for 12 hr resulted in the loss of 90% of PKC activity. Muscles treated with TPA showed an increased basal rate of 3-O-methylglucose uptake, responded partially to insulin but did not respond to contraction. For freshly isolated solei, insulin (10 mU/ml), increased the rate of 3-O-methylglucose uptake by 70% from 160 to 272 nmol/min/g and for electrical stimulation the rate increased by 89% to 302 nmol/min/g. Muscles incubated for 12 hr with no additions, 0.06% DMSO or 0.3% DMSO all responded to insulin and electrical stimulation to the same extent as freshly excised solei. Treatment with 1 or 5 uM TPA resulted in a partial loss of response to insulin which was down from 60-70% to 24-40%. A total loss of response to electrical stimuli was observed with treatment with 5 uM TPA. TPA (5uM) treatment for 12 hr also increased the basal rate of 3-O-methylglucose uptake by 41% from 160 to 225 nmol/min/g. TPA-treated and non-treated muscles were indistinguishable in terms of pre-contraction content of adenine nucleotide, creatine phosphate, lactate and glycogen as well as contractile performance and contraction induced glycogenolysis.

It is concluded that treatment of solei with TPA gives rise to a marked loss of contraction-induced glucose transport and that this may be due to loss of PKC.

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(Proc. Biochem. Soc. Aust. 21: sp14., 1989)

APPENDIX III

**PUBLICATIONS ARISING INDIRECTLY
FROM THIS THESIS**

**From: PLASMA MEMBRANE OXIDOREDUCTASES IN CONTROL
OF ANIMAL AND PLANT GROWTH**

Edited by Frederick L. Crane, D. James Morre, and Hans Low
(Plenum Publishing Corporation, 1988)

CARDIAC Ca^{2+} CHANNELS AND SARCOLEMMAL REDOX

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INTRODUCTION

Alpha-1-adrenergic receptors have been identified in both animal and human cardiac tissue (Schumann, 1978; Clark and Patten, 1984a; Bruckner et al., 1985) and several alpha adrenergic mediated events have been reported. Alpha agonists cause increases in inotropy of the heart (Bruckner et al., 1985) although this is not exclusive to alpha agonists as beta agonists also cause increases in inotropy and chronotropy. Alpha agonists also cause changes in metabolism such as activation of phosphofructokinase (Clark and Patten, 1984a) and increased glucose transport and uptake (Clark and Patten, 1984b; Rattigan et al., 1986; Abel et al., 1987), leading to increased glycolysis in the heart. Alpha stimulation has also been postulated to control hypertrophy of the heart (Simpson et al., 1986) and alpha agonists have been shown to cause the expression of c-myc and c-fos genes (Starksen et al., 1986; Barka et al., 1987).

The mechanism by which alpha adrenergic agonists mediate these events is not precisely known, but increases in myoplasmic Ca^{2+} concentration may be involved (Clark and Patten, 1984a; Rattigan et al., 1986). The increased Ca^{2+} appears to be due to the involvement of Ca^{2+} channels in the sarcolemma that are sensitive to Ca^{2+} channel blockers such as nifedipine (Bruckner et al., 1985; Clark and Rattigan, 1986). Beta agonists also regulate the same Ca^{2+} channels via phosphorylation of membrane components (e.g. calmodulin) by the cAMP-dependent protein kinase (Rinaldi et al., 1982). However it is apparent that alpha adrenergic stimulation does not involve cAMP or cAMP-dependent kinases (Clark and Patten, 1984a; Bruckner et al., 1985).

In other tissues it has become apparent that phosphoinositide (PI) hydrolysis occurs as a result of alpha-adrenergic stimulation (Berridge, 1982; Exton, 1985). Hydrolysis of PI leads to the formation of two second messengers, inositol 1,4,5 trisphosphate (IP_3) and 1,2 diacylglycerol (DAG). IP_3 has been shown to cause release of Ca^{2+} from intracellular stores and DAG causes the activation of protein kinase C (Berridge, 1982; Exton, 1985). As in the adenylate cyclase system there is evidence that a G-protein couples the alpha receptor to phospholipase C, the enzyme which hydrolyses PI. In some cells this G-protein appears to resemble the G_i subunit of the adenylate cyclase system in terms of

its sensitivity to pertussis toxin but is otherwise unique compared with G_i (Berridge, 1987).

In cardiac tissue PI hydrolysis and the formation of inositol phosphates in response to alpha stimulation has been shown in isolated duck, guinea pig and rat ventricular myocytes (Brown and Jones, 1987; Leung et al., 1986; Brown et al., 1985) and perfused rat heart (Woodcock et al., 1987). However production of DAG and activation of protein kinase C (PKC) have yet to be shown. It is unknown whether a G-protein is involved in the PI hydrolysis in the heart, but PI hydrolysis is not sensitive to pertussis toxin (Schmitz et al., 1987). A question also remains as to whether IP_3 can directly activate the sarcolemmal Ca^{2+} channels. Thus the mechanism of Ca^{2+} channel activation remains to be elucidated.

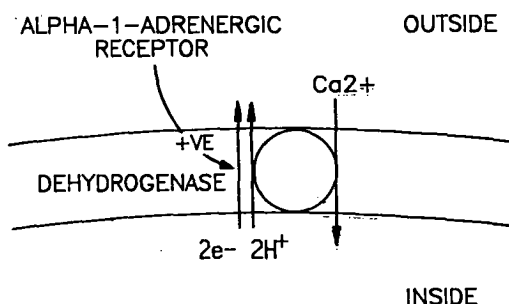


Figure 1. Proposed relationship between alpha-adrenergic effects on trans-sarcolemmal electron efflux and uptake of calcium through calcium channels in heart cells.

Transmembrane redox systems are present in all cells. Although no definitive role for this system has yet been established it has been shown that such systems are related to cell growth, facilitation of iron uptake and defense against bacteria, each of which involve specific ion movements (Crane et al., 1985). In the heart trans-sarcolemmal electron efflux, measured as extracellular ferricyanide reduction in perfused rat heart, is increased in response to alpha adrenergic stimulation (Low et al., 1984, 1985). Time and dose relationships indicate a close association between ferricyanide reduction and changes in contractility in the rat heart (Low, 1985; Clark and Rattigan, 1986). As changes in contractility reflect levels of myoplasmic Ca^{2+} concentration it has been postulated (Fig. 1) that the increase in reduction of extracellular ferricyanide results from an increase in electron efflux that is closely coupled to Ca^{2+} influx through sarcolemmal Ca^{2+} channels (Crane et al., 1985; Clark and Rattigan, 1986). Since NADH has now been identified as one of the probable intracellular donors for the trans-plasma membrane redox system (Navas et al., 1986) it appears likely that electron efflux is accompanied by proton efflux (Fig. 1).

Therefore in the present study the relationship of the PI signal transduction system and trans-sarcolemmal electron efflux in activating the sarcolemmal Ca^{2+} channels was investigated. Three possibilities were considered:

(1) alpha-adrenergic stimulation that causes PI hydrolysis, DAG formation and PKC activation causes the phosphorylation of proteins that increase trans-sarcolemmal electron efflux and opening of Ca^{2+} channels.

(2) alpha-adrenergic stimulation that causes PI hydrolysis and IP_3 formation causes opening of Ca^{2+} channels and stimulation of trans-sarcolemmal electron efflux mediated by IP_3 .

(3) alpha-adrenergic stimulation leads directly to increased trans-sarcolemmal electron efflux that opens Ca^{2+} channels without the involvement of PI hydrolysis.

These possibilities were investigated by using synthetic diacylglycerol and the phorbol ester, PMA, activators of protein kinase C (Berridge, 1982); compound C48/80 and neomycin, inhibitors of phospholipase C (Bronner et al., 1987; Downes and Michell, 1981; Whipps et al., 1987; Slivka and Insel, 1987); aluminium tetrafluoride, an activator of G-proteins involved in PI hydrolysis (Blackmore and Exton, 1986).

MATERIALS AND METHODS

Materials. L-phenylephrine, L-isoproterenol, DL-propranolol, phorbol-12-myristate-13-acetate (PMA), 4 α -phorbol 12,13-didecanoate (PDD), A23187, bathophenanthroline disulphonate, compound 48/80 and neomycin were obtained from Sigma. Diacylglycerol (1-oleoyl-2-acetyl glycerol) was obtained from either Serdary Research Laboratories or Sigma.

Heart Perfusion. Male Hooded Wistar rats (180-200 g) that had been fed ad libitum were used. Hearts were removed from the anaesthetized animals (pentobarbitone) and perfused in the Langendorff manner at 37°C with Krebs-Henseleit bicarbonate buffer pH 7.4 equilibrated against O_2 + CO_2 (95:5) and containing 1.25 mM CaCl_2 , 5 mM glucose and 0.5 mM sodium ferricyanide. After an equilibration period of 10 min experiments were commenced with perfusion being carried out in a non-recirculating manner at a constant perfusion pressure of 80 cm water. Agents were infused directly above the heart at a rate of 1/100th the perfusate flow rate.

Contractility was determined by the tension developed between two hooks placed 8 mm apart on the ventricle wall. Tension was measured by a isometric force transducer and initial (unstimulated) tension was set at 2.0 g peak to peak.

During experiments perfusate from the heart was collected into a fraction collector set on time mode (26 sec/fraction). Normally 10 fractions were collected before the agent was added (control rate) and then fractions were collected for a further 5 min after addition of the agent being investigated.

Assays. Samples of perfusate were taken and assayed for ferrocyanide using bathophenanthroline disulphonate (Avron and Shavit 1963). The rate of ferricyanide reduction (nmol/min/g wet wt. of heart) was calculated according to the following equation:

$$\text{Rate} = \frac{[\text{ferrocyanide}] (\mu\text{M}) \times \text{perfusate flow rate (ml/min)}}{\text{wt. of heart (g)}}$$

RESULTS

To investigate whether PKC was involved with increases in trans-sarcolemmal electron efflux and activation of Ca^{2+} channels, activators of PKC were added to the perfused rat heart. The effect of these activators on ferricyanide reduction and tension development are shown in Table 1.

TABLE 1. Effect of PKC activators on ferricyanide reduction and tension development in the perfused rat heart. Mean \pm S.E.M. (n).

Addition	Ferricyanide Reduction (% of preaddition rate) Δ	Increase in Tension (%)
Saline	92 \pm 6 (5)	0
Phenylephrine (20 μM) + Propranolol (10 μM)	153 \pm 13 (4)*	82
PMA (200 nM)	80 \pm 11 (3)	0
OAG (30 $\mu\text{g/ml}$)	102 \pm 3 (3)	0
A23187 (0.4 μM)	97 \pm 7 (3)	ND
OAG (30 $\mu\text{g/ml}$) + A23187 (0.4 μM)	111 \pm 9 (3)	ND

* significantly different from saline addition $P < 0.01$

ND - not determined

Δ preaddition rate of ferricyanide reduction was 60.3 ± 3.8 nmol/min/g wet wt. (n = 21)

Neither phorbol-12-myristate-13-acetate (PMA) or 1-oleoyl-2-acetyl glycerol (OAG) caused a stimulation of ferricyanide reduction. In some systems to elicit maximum responses the calcium ionophore A23187 is required with DAG (Kaibuchi et al., 1983) but this combination was also ineffective in the perfused heart (Table 1).

No sustained effect of PMA or OAG on tension development in the heart was observed (Table 1) but transient increases between 10 and 30 sec after the addition occurred. The magnitude of these increases were 22% for PMA and 27% for OAG.

Both PMA and OAG caused a decrease in perfusate flow rate and the time course for the PMA effects is shown in Figure 2. The decrease in flow rate caused by PMA was not reversible when PMA was removed.

The effects of the PKC activators on the alpha and beta adrenergic stimulation of the perfused heart are shown in Table 2. PMA but not OAG inhibited the alpha agonist (phenylephrine + propranolol) stimulation of ferricyanide reduction and contractility. PMA did not however block the increased contractility caused by the beta agonist isoproterenol. The inhibition of ferricyanide reduction caused by PMA was dose-dependent and appeared to be more sensitive than the reduction in perfusate flow caused by PMA (Figure 3).

The effect of agents that either stimulate G-proteins (NaF + AlCl₃) or inhibit phospholipase C (neomycin and C48/80) are shown in Table 3. NaF + AlCl₃ addition did not stimulate ferricyanide reduction and C48/80 treatment had no effect on ferricyanide reduction and contractility or on the alpha stimulation of these processes.

Neomycin was found to directly react with ferricyanide in the perfusate and thus its effect on ferricyanide reduction are unknown. Neomycin also had dramatic effects on contractility of the perfused heart leading to a virtual cessation of contraction. This effect was rapid and readily reversible with the removal of neomycin. Phenylephrine stimulation of the heart was able to overcome the inhibition of contraction caused by neomycin (results not shown).

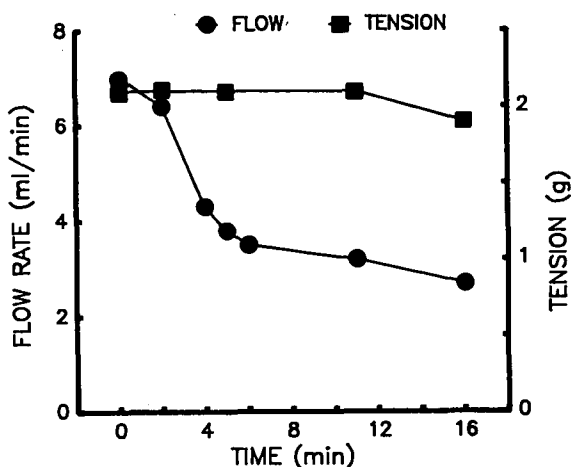


Figure 2. Time course for the effect of PMA (200nM) on flow rate and tension development in the perfused rat heart.

DISCUSSION

Alpha adrenergic stimulation of the perfused rat heart causes increased trans-sarcolemmal electron efflux that is closely associated with opening of sarcolemmal Ca²⁺ channels. This leads to increased myoplasmic Ca²⁺ concentration and contractility (Low et al., 1985; Clark and Rattigan, 1986). Alpha adrenergic stimulation in many tissues and in the heart causes the activation of the PI signal transduction system (Berridge, 1982; Exton, 1985; Woodcock et al., 1987). The PI signal transduction system has two branches, which involve the second messengers DAG and IP₃ (Berridge, 1982).

TABLE 2. Effect of PKC activators on alpha and beta adrenergic stimulated perfused rat heart. Mean \pm S.E.M. (n).

Pretreatment	Treatment	Ferricyanide Reduction (% of Pretreatment Rate) ^Δ	Increase in Tension Development (%)
None	Saline	91.6 \pm 5.9 (5)	0
"	Phenylephrine (20 μ M) + Propranolol (10 μ M)	153.3 \pm 12.9 (4)*	82
20' PMA (200 nM)	Phenylephrine (20 μ M) + Propranolol (10 μ M)	85.6 \pm 2.0 (3)	0
20' PDD (200 nM)	Phenylephrine (20 μ M) + Propranolol (10 μ M)	127.2 \pm 10.1 (3)*	ND
None	Isoproterenol (20 μ M)	NPD	73
PMA (200 nM)	Isoproterenol (10 μ M)	NPD	150
OAG (6 μ g/ml)	Phenylephrine (20 μ M) + Propranolol (10 μ M)	ND	82

ND - not determined

NPD - not possible to determine since agent directly reacts with ferricyanide

* significantly different from saline addition $P < 0.01$

^Δ pretreatment rate - 60.3 \pm 3.8 nmol/min/g wet wt. (n = 21)

The production of DAG in other tissues causes the activation of PKC (Berridge, 1982; Exton, 1985). Although it has not been shown that alpha adrenergic stimulation in the heart leads to production of DAG capable of activating PKC it has been shown that PMA, a direct activator of PKC, does cause the translocation of PKC from the cytosol to the particulate fraction in perfused rat heart (Davison et al., unpublished). Translocation is often considered an indication of activation of PKC (Nishizuka, 1984). In the present study addition of PMA did not cause any change to the trans-sarcolemmal electron efflux (Table 1). There was a transient rise in contractility, which might reflect Ca^{2+} release from intracellular stores (Exton, 1985), but no sustained increase in contractility occurred reflecting that sarcolemmal Ca^{2+} channels were not activated. The synthetic diacylglycerol, OAG, another activator of PKC in other systems (Evans and Farrar, 1987), was also ineffective, whether used alone or in combination with the calcium ionophore A23187 (Table 1).

Both PMA and OAG did decrease the perfusate flow rate (Figure 2) indicating they were capable of causing a physiological response in the perfused heart system. Also PMA pretreatment caused a loss of the alpha adrenergic stimulation of trans-sarcolemmal electron efflux and contractility (Table 2) in a dose-dependent manner (Figure 3). In other tissues PKC activation has been shown to cause phosphorylation and down-regulation of the alpha adrenergic response (Cotecchia et al., 1985; Carvera et al., 1986; McMillan et al., 1986; Woods et al., 1987; Kunos and Ishac, 1987). This may occur at the receptor level or at the

G-protein that couples the receptor to the response (Kunos and Ishac, 1987; Orellana et al., 1987).

The results of the present study indicate that PKC activation is not involved in the stimulation of trans-sarcolemmal electron efflux or opening of the sarcolemmal Ca^{2+} channels. However PKC may regulate the alpha adrenergic stimulation of these responses by down-regulation at the receptor level.

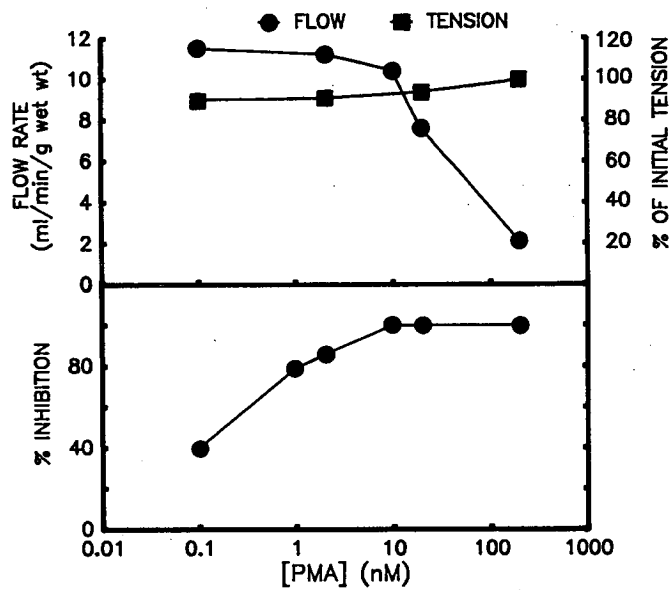


Figure 3. Dose curve for the effect of PMA on flow rate, tension development and the inhibition of the phenylephrine stimulation of ferricyanide reduction in the perfused rat heart.

The other second messenger of the PI signal transduction system is IP_3 . IP_3 production can be blocked in other tissues by inhibition of phospholipase C with neomycin (Downes and Michell, 1981; Whipps et al., 1987; Slivka and Ingel, 1987) or with C48/80 (Bronner et al., 1987). The effect of neomycin addition to the perfused heart was complex. Contractility was rapidly decreased indicating that neomycin was acting as more than just an inhibitor of phospholipase C. As neomycin reacted directly with ferricyanide in the perfusate it was not possible to assess its effects on trans-sarcolemmal electron efflux. Alpha adrenergic stimulation was able to overcome the effects of neomycin on the contractility of the heart suggesting that phospholipase C activation and thus IP_3 production was not necessary for sarcolemmal Ca^{2+} channel activation. This was further supported by the observation that C48/80 was also ineffective in blocking the alpha adrenergic stimulation of contractility and trans-sarcolemmal electron efflux (Table 3). However

TABLE 3. Effect of NaF + AlCl₃, neomycin and C48/80 on ferricyanide reduction and tension development in the perfused heart.

Pretreatment	Treatment	Ferricyanide Reduction (% of Control) ^φ	Tension Development (% of Control)
None	NaF (5 mM) + AlCl ₃ (10 μM)	104.7	ND
"	Neomycin (2 mM)	677 ^Δ	<10
"	C48/80 (5 μg/ml)	89.4	75
C48/80 (5 μg/ml)	Phenylephrine (20 mM) + Propranolol (10 μM)	125 ± 7 (3)*	140 ± 10 (3)*

^Δ neomycin reacted directly with ferricyanide

* significant increase P<0.01

^φ control rate 60.3 ± 3.8 nmol/min/g wet wt. (n = 21)

conclusive evidence that IP₃ is not involved awaits studies showing that IP₃ production in the heart is blocked by these agents.

In conclusion these results indicate that the PI signal transduction system is not likely to be involved in the stimulation of either trans-sarcolemmal electron efflux or activation of sarcolemmal Ca²⁺ channels.

Recently Yatani et al. (1987) have shown that a G-protein can directly regulate mammalian cardiac Ca²⁺ channels. Aluminium tetrafluoride causes activation of the G-protein coupled to the alpha adrenergic receptor in liver tissue (Blackmore and Exton, 1986). Thus NaF + AlCl₃ were added to the perfused heart, but no effect on trans-sarcolemmal electron efflux was observed (Table 3). This may be due to the cardiac G-protein being different to the liver G-protein and thus not being sensitive to aluminium tetrafluoride. It is already known that pertussis toxin does not inhibit the alpha adrenergic mediated effect on IP production in the heart unlike the inhibition that occurs in liver (Schmitz, 1987).

The mechanism by which alpha adrenergic stimulation of the rat heart causes the activation of trans-sarcolemmal electron efflux and the sarcolemmal Ca²⁺ channels as well as the relationship between each await further research.

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EFFECT OF PHORBOL ESTERS ON THE DISTRIBUTION AND TOTAL ACTIVITY OF PROTEIN KINASE C IN THE PERFUSED RAT HEART

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Abstract—1. The perfused rat heart was treated with the tumour-promoter and protein kinase C activator, phorbol 12-myristate 13-acetate and the distribution of protein kinase C activity between cytosolic and particulate fractions determined.

2. Phorbol ester treatment led to a rapid loss of protein kinase C activity from the cytosol ($t_{0.5} = 2$ min) with a corresponding translocation into the particulate fraction. Translocated protein kinase C activity was tightly bound to the particulate fraction, could only be extracted with buffers containing 2% Triton X-100 and could therefore be misinterpreted as being down-regulated.

3. Claims of rapid down-regulation of protein kinase C activity by phorbol esters need to be supported by rigorous procedures for extraction of the particulate material.

INTRODUCTION

Protein kinase C (PKC), the phospholipid, Ca^{2+} -dependent protein kinase is present in heart (Kuo *et al.*, 1980), but its precise role is still not understood (see Brown and Brown, 1986). Tumour-promoting phorbol esters bind to, and activate PKC in most tissues and have been used to assess the role of PKC in cellular events.

PMA effects on heart preparations include an increase in Na^+/H^+ exchange resulting in cytosolic alkalization of chick cardiac cells (Green *et al.*, 1986) and PMA in combination with the Ca^{2+} ionophore, A23187, induces secretion of atrial natriuretic peptide (Ruskoaho *et al.*, 1985). PMA has also been reported to affect calcium and potassium channels in isolated heart cells (Lacerda *et al.*, 1988; Walsh and Kass, 1988) and synthetic diacylglycerol or PMA treatment also decreases β -adrenergic receptors in rat cardiac myocytes (Limas, 1980).

Contractile effects of PMA appear to be rather contradictory. Ruskoaho *et al.* (1985) reported that PMA increased rate and decreased tension in the perfused heart preparation. Other reports suggest that PMA has both negative inotropic and chronotropic effects (Leatherman *et al.*, 1985; Capogrossi *et al.*, 1986; Hansford *et al.*, 1986) while we have previously reported a transiently increased tension development in the perfused rat heart (Clark *et al.*, 1988).

Phorbol esters have been extensively used to activate PKC in intact cell systems in short-term studies and to down-regulate PKC in similar systems over

longer periods (usually 24 hr or greater). However some reports suggest that short-term (minutes) exposure may be sufficient to achieve down regulation (Green *et al.*, 1986; Issandou *et al.*, 1986; Shenolikar *et al.*, 1986; Cooper *et al.*, 1987; Ishizuka *et al.*, 1987; Nagao *et al.*, 1987). Activation of PKC with PMA is usually observed as a translocation of PKC from the cytosol to the particulate fraction. However no studies have been reported for the effects of PMA on the perfused heart system. Thus in this study, rat hearts were perfused with PMA to assess the effects on the distribution and total activity of PKC.

MATERIALS AND METHODS

Perfusions

Male hooded Wistar rats weighing 200–250 g were used for these experiments. Hearts were perfused in the Langendorff manner using a system based on that of Williamson (1964) and described elsewhere (Clark and Patten, 1984). The perfusion medium was Krebs–Henseleit bicarbonate buffer and unless indicated otherwise contained 1.27 mM CaCl_2 , 0.05 mM EDTA and 5 mM glucose. The perfusion temperature was 37°C and the medium was equilibrated with CO_2/O_2 (1:19). The hearts were preperfused at a flow rate of 5–8 ml/min for 10 min in a non-recirculating manner prior to commencing the experiment.

Phorbol esters or synthetic diacylglycerols were infused as concentrated solutions directly above the heart. PMA was initially dissolved in dimethyl sulphoxide then diluted in 10% (v/v) dimethyl sulphoxide/saline for infusion. Perfusions involving PMA were conducted using equipment shielded from direct light. Control perfusions involved the infusion of 10% dimethyl sulphoxide (0.1% final concentration).

Homogenization

Since the distribution of PKC can be altered by the free Ca^{2+} concentration during homogenization (Gopalakrishna *et al.*, 1986) the hearts were homogenized in a Ca^{2+} /EGTA

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Abbreviations: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PDD, 4- α -phorbol 12,13-didecanoate.

buffered solution (Burgess *et al.*, 1983). Hearts were homogenized in 4 ml of ice-cold buffer (pH 7.2) containing 250 mM sucrose, 1 mM dithiothreitol, 5 mM MgSO_4 , 0.96 mM NaH_2PO_4 , 25 mM NaHCO_3 , 10 mM EGTA, 5.5 mM CaCl_2 which was gassed with CO_2/O_2 (1:19) prior to and during homogenization. The $[\text{Ca}^{2+}]_{\text{free}}$ of this buffer was 240 nM and was unaffected by the addition of the heart as shown by Quin II fluorescence (data not shown).

Partial purification of PKC

The extraction and partial purification of PKC was essentially as described by Kikkawa *et al.* (1982). In brief, the crude homogenate was centrifuged at 100,000 *g* for 60 min and the resultant supernatant applied directly to a 4 ml column of Whatman DE-52 cellulose. The pellet (particulate fraction) was resuspended in 6 ml of extraction buffer which contained 20 mM Tris-HCl pH 7.2, 2 mM Na_2EDTA , 0.5 mM EGTA, 1 mM dithiothreitol and 0.2% Triton X-100. After an extraction period of 60 min at 0–4°C and intermittent mixing, the suspension was centrifuged at 100,000 *g* for 30 min. The supernatant was applied to a second DE-52 anion exchange column and the extraction procedure repeated with 6 ml of extraction buffer which contained 2% Triton X-100. The second supernatant was

applied to a third DE-52 column. These conditions were found to extract all the PKC from particulate fractions of control hearts.

Anion exchange chromatography was adapted from the method of Averdunk and Gunther (1986). Columns were loaded with 4 ml of preswollen DE-52 cellulose and equilibrated with 20 ml of column buffer containing 20 mM Tris-HCl pH 7.5, 2 mM Na_2EDTA , 0.5 mM EGTA and 1 mM dithiothreitol at 5°C. Cytosolic or Triton X-100 extracts of the particulate fraction (*ca* 25 mg protein) were applied to the columns, washed with 20 ml of column buffer then eluted with a linear gradient of 40 ml of 0–150 mM NaCl in column buffer at 1 ml/min. Other gradients were used as indicated in the relevant figures. Fractions (4 ml) were collected for assay.

PKC assay

PKC activity in the column fractions were assayed by measuring the incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into histone H1S. The reaction mixtures (final volume 120 μl) contained 3.8 μmol Tris-HCl at pH 7.5, 1.25 μmol magnesium acetate, 50 μg of histone H1S, 2.5 nmol of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ($5\text{--}10 \times 10^4$ cpm/nmol) and either 8 μg phosphatidyl serine, 0.16 μg diolein and 250 nmol of calcium

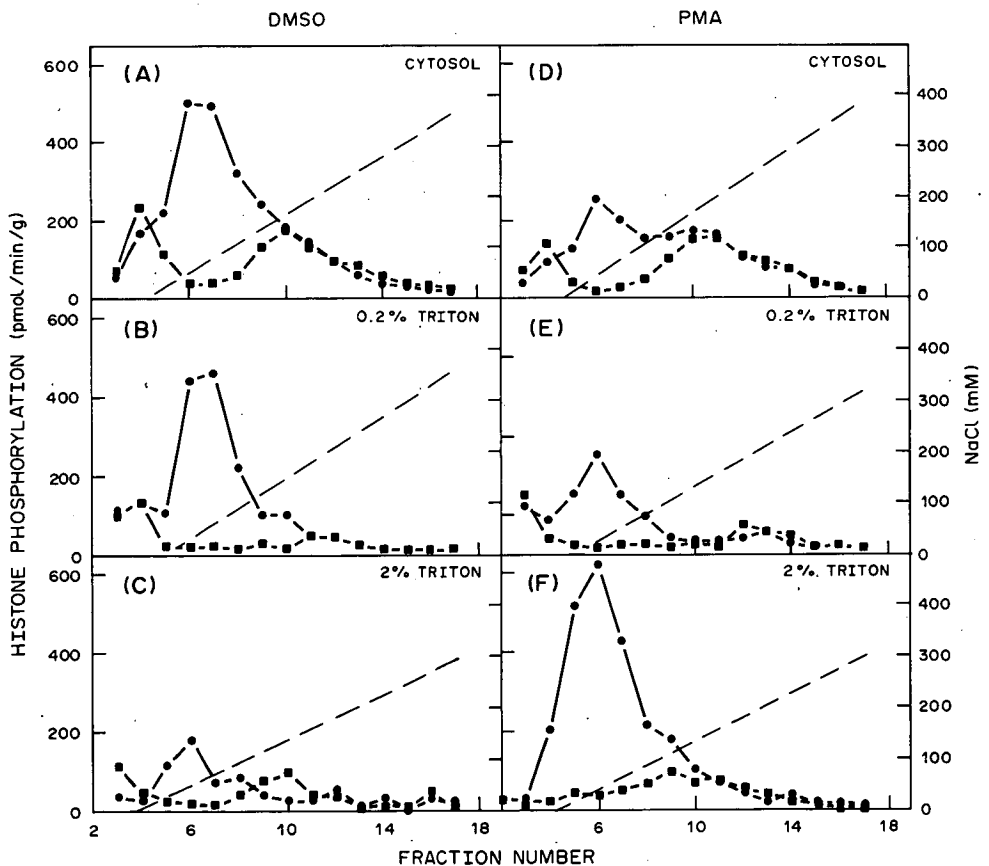


Fig. 1. Effect of phorbol ester treatment of perfused rat heart on the cytosolic and particulate PKC activities. Hearts were perfused for 10 min in a non-recirculating mode with medium containing 5 mM glucose, 1.27 mM Ca^{2+} and 0.05 mM EDTA and then for 10 min with the same medium (A–C) or the same medium containing 200 nM PMA (D–F). Hearts were then immediately homogenized in buffer containing 10 mM EGTA and Ca^{2+} at a ratio of 0.55:1 to give a free Ca^{2+} concentration of 240 nM. Homogenates were centrifuged at 100,000 *g* for 1 hr. The pellet was extracted with 0.2% Triton X-100 (B, E) and then with 2.0% Triton X-100 (C, F) and protein kinase activity was resolved by anion exchange chromatography as described in the Materials and Methods section. The supernatant was applied directly to anion exchange columns (A, D). Column fractions were assayed for histone phosphorylating activity in the presence of Ca^{2+} , diolein and phosphatidyl serine (●) or in the presence of EGTA alone (■). The NaCl gradient is depicted by the broken line.

chloride in the active mixture or 250 nmol of EGTA in the basal assay mixture. The reaction was commenced by addition of 50 μ l of column fractions and after incubation at 30°C for 12 min, 50 μ l aliquots were pipetted onto 2 \times 2 cm squares of phosphocellulose P81 paper. The papers were washed twice in 5% trichloroacetic acid and once in water before drying and counting for radioactivity. The activity of PKC was expressed as the difference between active and basal assays and given as pmoles of 32 P incorporated per min.

Considerable variation in the rate of histone phosphorylation was noted between each batch of histone IIIS and occasionally between bottles of the same batch. To remove this variation supplies of histone were combined, dissolved in distilled water and lyophilized.

Protein kinase M

Partial proteolytic digestion of PKC to protein kinase M was conducted essentially as described by Ferrari *et al.* (1987). Column fractions from anion exchange chromatography containing high PKC activity and eluting at ca 50 mM NaCl were pooled and a 10 ml aliquot was incubated with 10 ml of 20 mM Tris-HCl buffer pH 8.0 containing 10 mg/ml bovine serum albumin and 8 μ g/ml trypsin. The mixture was incubated at 30°C for 6 min and terminated by the addition of 1 ml of 0.5 mg/ml soya bean trypsin inhibitor and immersion into ice. The digested sample was loaded onto a DE-52 anion exchange column and the kinase activity resolved using a 60 ml linear gradient of 0–0.5 M NaCl in column buffer.

Kinase activity eluting at ca 200 mM NaCl (putatively protein kinase M) was assayed with histone IIIS as described. Protein kinase M was found to be neither Ca^{2+} nor phospholipid-dependent for the phosphorylation of histone.

Materials

Histone IIIS, diolein, phosphatidyl serine, PMA, 1-oleoyl-2-acetyl *rac* glycerol (OAG), 1,2-dicapryloyl-*rac*-glycerol (DiC8), 4- α -phorbol 12,13-didecanoate (PDD), trypsin and soya bean trypsin inhibitor were obtained from Sigma Chemical Co. Preswollen DE-52 cellulose and phosphocellulose P81 paper were from Whatman. [γ - 32 P]ATP was supplied by Bresatec Pty. Ltd, Adelaide, Australia.

RESULTS AND DISCUSSION

PMA is used both to activate and to down-regulate PKC. Figure 1 shows the effect of 10 min perfusion with 200 nM PMA on the anion exchange column profile of histone kinase activity of the cytosolic, 0.2% and 2% Triton X-100 extracts of the particulate material from hearts homogenized in a 240 nM Ca^{2+} buffered solution. PMA produced a marked decrease in the kinase activity peak eluting at 50 mM NaCl in both the cytosolic and 0.2% Triton X-100 extract but an increase in the 2% Triton X-100 extract of the particulate fraction. This activity was characteristic of PKC and eluted at an identical position to purified porcine brain PKC (results not shown). Column fractions were used to determine the total PKC activity in the supernatant (cytosol) and in the particulate extracts of control and PMA treated hearts. These are shown in Table 1 and indicate that an apparent down regulation of PKC activity has occurred when the less rigorous 0.2% Triton X-100 extraction conditions are used.

Figure 2 shows the time course for the loss of PKC activity from the cytosolic and 0.2% Triton X-100 extract of particulate material reduced by perfusion

Table 1. Effect of extraction procedures on the activity of PKC associated with the particulate fraction

	PKC activity (pmol/min g wet wt)	
<i>Supernatant</i>		
Control	1156 \pm 134 (7)	
PMA	342 \pm 61 (7)*	$P < 0.001$
<i>Particulate—0.2% Triton</i>		
Control	1610 \pm 210 (3)	
PMA	872 \pm 297 (3)	
<i>Particulate—2% Triton</i>		
Control	2368 \pm 320 (4)†	NS
PMA	3211 \pm 461 (4)†	$P < 0.02$

Homogenates of control hearts of hearts perfused with 200 nM PMA (10 min) were prepared as described in the Materials and Methods section. The particulate material was extracted with buffer containing the concentrations of Triton X-100 as shown. Results are means \pm SEM with the number of heart perfusions shown in parentheses. Statistical difference was determined using the student's *t*-test; *PMA vs control, †2% Triton vs 0.2% Triton; NS, not significant.

with 200 nM PMA (homogenization in 240 nM Ca^{2+} buffer). Both cytosolic activity and 0.2% Triton X-100 extracts were decreased by 60% by 5 min with an apparent $t_{0.5}$ of 2 min. Figure 2 also shows that PKC activity did not return to pretreatment values following removal of the PMA for 20 min.

The dose-dependent relationship for the translocation of the PKC activity is shown in Fig. 3. Hearts were perfused for 5 min with PMA and the

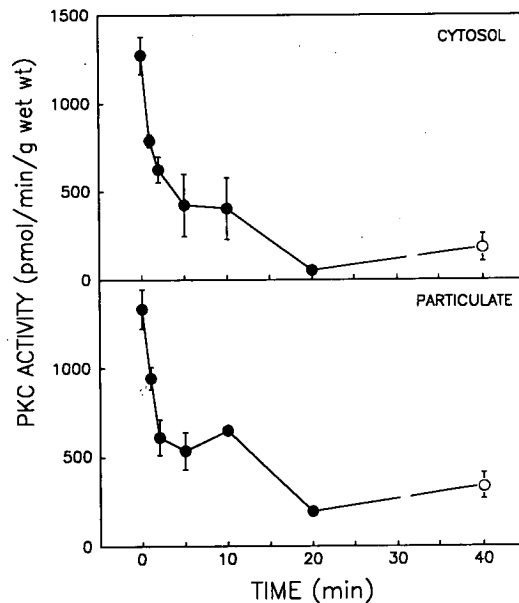


Fig. 2. Time course for the effect of phorbol ester on PKC activities of perfused rat heart. Hearts were perfused for 10 min in a non-recirculating mode with medium containing 5 mM glucose, 1.27 mM Ca^{2+} and 0.05 mM EDTA and then with medium containing 200 nM PMA for the times shown (●). In addition some hearts that were perfused for 20 min with 200 nM PMA were changed back to medium containing no PMA for a further 20 min (○). Hearts were treated as described for Fig. 1 and in the Materials and Methods section. PKC activity was determined on fractions following anion exchange chromatography of the supernatant and 0.2% Triton X-100 extracts of the particulate material. Where indicated means \pm range are given for duplicate perfusions.

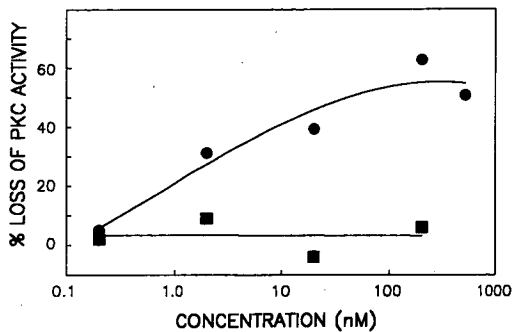


Fig. 3. Dose curve for the effect of phorbol ester on total PKC activity of perfused rat heart. Hearts were perfused for 10 min in a non-recirculating mode with medium containing 5 mM glucose, 1.27 mM Ca^{2+} and 0.05 mM EDTA and then for 5 min with medium containing PMA (●) or PDD (■) at the concentrations shown. Hearts were treated as described for Fig. 1 and the Materials and Methods section. PKC activity was determined on fractions following anion exchange chromatography of the supernatant and 0.2% Triton X-100 extracts of the particulate material. Values are from single hearts.

particulate fraction extracted with 0.2% Triton X-100 after homogenization in 240 nM Ca^{2+} buffer. Under these conditions half-maximal loss of PKC activity from cytosolic and 0.2% Triton extracts occurred at *ca* 10 nM PMA. There was no effect of the non-tumour promoting phorbol ester, PDD (Fig. 3).

In some tissues treatment with phorbol esters leads to translocation of PKC to the membrane fraction followed by proteolytic cleavage to a

non- Ca^{2+} -dependent form (Tapley and Murray, 1985). This latter enzyme elutes from anion exchange resin at a higher NaCl concentration than PKC, is generally found in the cytosolic fraction and has been named protein kinase M (Kishimoto *et al.*, 1983). Experiments were thus conducted to assess whether proteolytic modification had occurred. Anion exchange columns were standardized using protein kinase M generated by mild trypsin treatment of purified PKC; fractions eluting at *ca* 200 mM NaCl were found to contain activity consistent with protein kinase M. No increase in kinase activity eluting at 200 mM NaCl was observed in either cytosolic or particulate fractions of PMA treated hearts (Fig. 1) and all the activity could be recovered from the 2% Triton X-100 extracts (Table 1).

In contrast to PMA, the diacylglycerol, DiC8 (200 $\mu\text{g}/\text{ml}$) had no effect on the distribution of PKC between the cytosolic and particulate fractions of the perfused heart and the distribution was *ca* 50% in each fraction. Recovery of PKC activity using 0.2% Triton X-100 was the same as controls extracted under the same conditions (data not shown). These results indicate that the effect PMA has on the heart is different from that which occurs with agonists that stimulate phosphoinositide hydrolysis.

Finally, the effect of PMA treatment on PKC activity has been investigated in a number of tissues and cells recently and it is of interest to compare the methods of extraction as well as the results obtained (Table 2). Inspection of Table 2 shows that either PKC is translocated from the soluble cytosolic fraction to the particulate fraction where it is sometimes degraded (down-regulated) or in some cases appears

Table 2. Comparison of extraction procedures for solubilizing particulate PKC from PMA-pretreated tissue

Detergent	EGTA (mM)	Tissue	Finding	Reference
<i>Triton X-100 (%)</i>				
0.1	1	Guinea pig parietal cell	DR	Beil <i>et al.</i> (1987)
0.1	5	Brain slices	DR	Shenolikar <i>et al.</i> (1986)
0.1	0.5	U937 cells	DR	Ways <i>et al.</i> (1987)
0.1	5	Adrenal chromaffin cells	T	Terbush and Hotz (1986)
0.1	10	β -lymphocytes	T	Nel <i>et al.</i> (1986)
0.2	1	HL 60 cells	T	Shoji <i>et al.</i> (1986)
0.2	0	Thyroid cells (3 mM EDTA)	DRT	Omri <i>et al.</i> (1988)
0.3	2	BCH3H-1 myocytes	T	Spach <i>et al.</i> (1986)
0.3	2.5	Rat liver	T	Buckley <i>et al.</i> (1987)
0.5	10	MCF-7	T	Darbon <i>et al.</i> (1986)
0.5	10	Swiss 3T3 cells	DRT	Pelech <i>et al.</i> (1986)
0.5	10	Human breast cancer cells	DRT	Issandou <i>et al.</i> (1986)
1.0	10	Fibroblasts	DR	Cochet <i>et al.</i> (1986)
1.0	5	BC3H-1 myocytes	DR	Cooper <i>et al.</i> (1987)
1.0	0	Rat hepatocytes (2 mM EDTA)	T	Hernandez-Stotomayer and Garcia-Sainz (1988)
1.0	10	Perfused heart	DRT	Van de Werve <i>et al.</i> (1987)
1.5	0	HL-60	T	May <i>et al.</i> (1985)
2.0	10	Human T lymphocytes	T	Larsen <i>et al.</i> (1988)
<i>Nonidet NP-40 (%)</i>				
0.5	0	Guinea pig synaptosomes (2 mM EDTA)	DRT	Diaz-Guerra <i>et al.</i> (1988)
1.0	0.5	Rat adipocytes	T	Glynn <i>et al.</i> (1986)
1.0	2	Mast cells	DR	Nagao <i>et al.</i> (1987)
1.0	0.5	Human leukemic cells	T	Homma <i>et al.</i> (1986)
1.0	0	Rat hepatocytes	T	Vaartjes <i>et al.</i> (1986)
1.0	2	Pancreatic acinar cells	DR	Ishizuka <i>et al.</i> (1987)

DR—loss of total (soluble and particulate) PKC activity which occurred within minutes with no net translocation into the particulate fraction. DRT—loss of total PKC activity but with some increase in the particulate fraction. T—translocation of PKC activity quantitatively from the soluble to the particulate fraction. In each report the particulate material appeared to be extracted only once with detergent-containing buffer.

to be rapidly down-regulated without translocation. The conditions used to extract PKC from the particulate fraction vary considerably (see Table 2) and the use of differing conditions within a single study for the extraction of PKC from control and PMA treated systems are rarely or never attempted. In this series of experiments we observed that total PKC activity could be extracted from control perfused heart particulate fractions with 0.2% Triton X-100 but that PMA treated hearts required 2% Triton X-100 extraction of particulate fractions to recover the enzyme activity (Fig. 1 and Table 1).

In conclusion we report that PMA treatment of the perfused rat heart leads to a rapid translocation of PKC from the cytosol to the membrane fraction. The tightly bound enzyme thus resulting can be misinterpreted as down-regulated unless rigorous extraction procedures are used to recover the activity.

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Glucose-Induced Loss of Exercise-Mediated 3-O-Methyl Glucose Uptake by Isolated Rat Soleus and Epitrochlearis Muscles

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Introduction

Contraction of muscle is associated with a marked increase in glucose uptake (Wallberg-Henriksson 1987 and references therein) and skeletal muscle is the major site of insulin-stimulated glucose disposal (James, Burleigh and Kraegen 1985). There are similarities between the effects of insulin and exercise to increase skeletal muscle glucose uptake (James, Kraegen and Chisholm 1985; Wallberg-Henriksson 1987), but the mechanisms are likely to be separate as additivity of the two occurs for red muscle (Ploug, Galbo, Vinten, Jorgensen and Richter 1987) and may also occur for white muscle (Wallberg-Henriksson 1987). In addition it is not clear whether insulin resistance is always accompanied by exercise-resistance in describing muscle glucose uptake. In diabetics, *in vivo*, muscle shows a poor response to insulin but responds normally to exercise (Wahren, Hagenfeldt and Felig 1975). However, muscle from insulin-deficient diabetic rats shows a marked decrease in responsiveness to both insulin and contraction-induced glucose transport (Wallberg-Henriksson 1987). Recent reports that hyperglycemia in the rat *in vivo* (Rossetti, Smith, Shulman, Papachristou and DeFronzo 1987), in type I diabetes (Yki-Jarvinen, Helve and Koivisto 1987), and in the perfused rat hindlimb (Richter, Hansen and Hansen 1988) induce a state of insulin resistance pose the question whether contraction-induced glucose transport is also impaired. To address this issue, soleus and epitrochlearis muscles were incubated with normal (5 mM) and a high (20 mM) concentration of glucose and, after washing, their ability to take up 3-O-methyl glucose in response to maximal insulin, electrical stimulation and a combination of the two was determined.

Materials and Methods

Female hooded Wistar rats (66–65 g for soleus; 100–120 g for epitrochlearis) were anaesthetized with pentobarbital i. p. (5 mg). The soleus, comprising 84% slow oxidative and 16% fast oxidative glycolytic fibres (Ariano, Armstrong and Edgerton 1973) and epitrochlearis, comprising 8% slow oxidative, 17% fast oxidative glycolytic and 75% fast glycolytic fibres (Wallberg-Henriksson 1987) muscles were removed and attached via the tendons to stainless steel U-shaped supports (15 mm in width) designed to maintain a tension of approx. 10 g. Muscles were incubated with shaking (110 oscillations \cdot min⁻¹) in pairs at 30 °C for 12 h in stoppered 50 ml flasks which contained 5 ml of incubation medium. Prior testing showed that a 12 h incubation period allowed extended exposure to high glucose concentration without significant loss of integrity (see below). The incubation medium consisted of saline-bicarbonate buffer (93 mM NaCl, 5 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 0.05 mM Na₂ EDTA, 1.27 mM CaCl₂, and 25 mM NaHCO₃) gassed with 95% O₂-5% CO₂ to give a pH of 7.4 and 0.1% freeze-dried serum from healthy rats. The total glucose concentration was either 5 or 20 mM; other additions included 35 mM mannitol (with 5 mM glucose) or 20 mM mannitol

(with 20 mM glucose) and 10 μ U \cdot ml⁻¹ insulin. The flasks were continuously gassed with O₂-CO₂ and the medium was changed every 4 h. For 3-O-methyl glucose uptake determinations, the muscles were removed from the primary incubations after 12 h, washed in glucose-free and insulin-free incubation medium and remounted by the tendons in a muscle suspension apparatus similar to that described by Nesher, Karl, Kaiser and Kipnis (1980). The apparatus contained 4 ml of modified Krebs-Ringer-bicarbonate buffer (KRB): 93 mM NaCl, 5 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 0.05 mM Na₂ EDTA, 1.27 mM CaCl₂, 25 mM NaHCO₃, 5 mM HEPES, 5 mM pyruvate, 8 mM (epitrochlearis) or 50 mM (soleus) 3-O-methyl-[1-³H]glucose (0.4 μ Ci \cdot ml⁻¹) and 5 mM [U-¹⁴C]sucrose (0.125 μ Ci \cdot ml⁻¹). The apparatus was maintained at 37 °C and was gassed with O₂-CO₂ to give a pH of 7.4. Gas flow was broken into small bubbles to provide thorough mixing of the KRB. Two muscles were mounted simultaneously and details for electrical stimulation (Hugo Sachs Electronic Nerve Muscle Stimulator Type 214) were: 200 ms train of impulses (each impulse of 1 ms duration) at a frequency of 50 Hz every 2 s and the voltage was adjusted (10–20 V) to attain full fibre recruitment. Tension development was recorded using a Harvard Bioscience Isometric Transducer. Muscles were frozen (liquid N₂) and then homogenized in 2 ml H₂O (Ultra-Turrax, 2 \times 30 s), centrifuged (15 min, 1500 \times g) and 1.5 ml of the supernatant was added to 12 ml ACS II (Amersham) for counting. Inclusion of [U-¹⁴C]sucrose in the incubations enabled correction for the extracellular space. ATP, ADP, AMP, creatine phosphate and lactate were determined in neutralized perchlorate extracts of frozen muscle. The adenine nucleotides were determined by HPLC (Waters) using a Pharmacia Mono Q anion exchange column. AMP, ADP and ATP were eluted in order using a linear gradient from 4.5 to 150 mM ammonium sulphate in 20 mM Tris-HCl pH 8.0 and detected at 254 nm. Creatine phosphate, lactate and glycogen were determined using standard methods (Bergmeyer 1974).

Results and Discussion

Muscle viability and integrity was assessed at the end of the 12 h (30 °C) incubation by comparing metabolite concentrations and by comparing tension development during electrical stimulation with freshly excised muscles. Values for soleus and epitrochlearis muscles (n = 3) after 12 h incubation were 23.6 \pm 3.5 and 27.4 \pm 0.8 (ATP); 3.1 \pm 0.5 and 5.2 \pm 1.0 (ADP); 1.9 \pm 0.1 and 1.1 \pm 0.5 (AMP); 40.6 \pm 2.0 and 56.8 \pm 4.6 (creatine phosphate); 16.0 \pm 2.2 and 8.2 \pm 1.06 (lactate); 98.1 \pm 21.1 and 138.9 \pm 2.1 (glycogen) μ mol \cdot g⁻¹ dry wt respectively. Water content of soleus and epitrochlearis muscles after 12 h incubation were 853 \pm 8.1 and 832 \pm 10.1 μ l \cdot g⁻¹ wet wt, respectively. None of these values differed significantly from those of fresh muscle. In addition developed tension (during the 30 min 3-O-methyl glucose uptake incubation) was the same for muscles that had been incubated at 30 °C for 12 h as those that had been freshly excised. The principal findings from the present

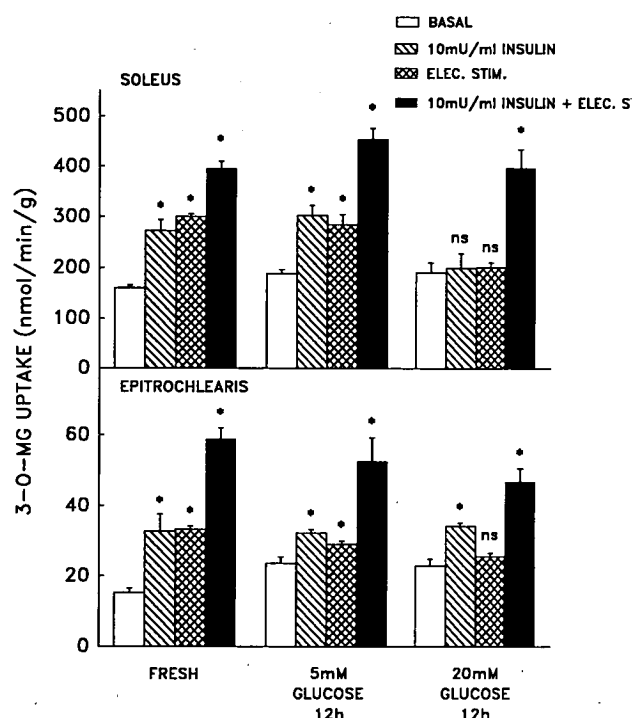


Fig. 1 3-O-Methyl glucose uptake of isolated soleus and epitrochlearis muscles, freshly excised ($n = 3$ pairs), and pre-incubated for 12 h at 30 °C with 5 mM or 20 mM glucose ($n = 5$ pairs for each group). Uptake of 3-O-methyl [$1-^3$]glucose was determined after 30 min incubation at 37 °C for resting muscles with no additions (basal), resting muscles with 10 mU/ml insulin, and muscles undergoing electrical stimulation (electrical stimulation with and without 10 mU/ml insulin). * Significantly different from basal ($P < 0.05$), n. s. not significantly different ($P > 0.05$) by Student's t-test.

study were that contraction-induced glucose uptake was markedly impaired in both muscle types that had been incubated for 12 h with 20 mM glucose and that resistance to contraction-induced glucose uptake was overcome by the presence of insulin (Fig. 1). In addition it was noted that insulin responsiveness by isolated soleus muscles was also lost but epitrochlearis muscles were unaffected. These latter observations suggest a partial dissociation of the effects of a high glucose load on insulin-induced glucose uptake in these two muscle types where previously a 5 h perfusion with less glucose (11–13 mM) affected red and white fibre-containing muscles similarly (Richter, Hansen and Hansen 1988). For both muscle types incubation for 12 h with 20 mM glucose led to an increase in the basal rate of glucose uptake; this was greatest for epitrochlearis muscles but less than that reported by Young, Uhl, Cartee and Holloszy (1986). Overall the present findings extend the observations made recently by Richter, Hansen and Hansen (1988) that prolonged exposure of the perfused rat hindlimb

to moderate levels of glucose induces a state of insulin resistance by showing that a state of "exercise-resistance" also occurs. This may not be entirely unexpected as recent studies indicate that insulin and exercise may in part use similar mechanisms where both lead to an increase in glucose transporters in the plasma membranes of rat skeletal muscle (Hirshman, Wallberg-Henriksson, Wardzala, Horton and Horton 1988; Douen, Ramlal, Klip, Young, Cartee and Holloszy 1989) even though exercise is unable to decrease the number of transporters in the insulin-sensitive intracellular pool (Douen, Ramlal, Klip, Young, Cartee and Holloszy 1989). The finding that both states of resistance induced by glucose loading in the present study can be overcome by the combination of muscle contraction with insulin further supports the view that certain aspects of the mechanism are shared.

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POSTER

P35 Ca^{2+} -MEDIATED TRANSLOCATION OF PROTEIN KINASE C AND APPARENT DOWN-REGULATION BY PHORBOL ESTER IN PERFUSED RAT HEART

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Protein kinase C, the phospholipid-, Ca^{2+} -dependent protein kinase is present in heart but its precise role is still not understood (1). Several specific cardiac proteins serve as substrates for protein kinase C *in vitro*. These include troponin I, troponin T, phospholamban, cardiac C-protein, phosphofructo-2-kinase as well as several unidentified proteins in sarcoplasmic reticulum and sarcolemma (see ref. 1 and references therein). Whether these protein substrates are directly involved in the implied actions of protein kinase C of control of hypertrophy (2), glucose transport (3), or other events appears uncertain but related substrates for protein kinase C in non-cardiac tissues are ribosomal S_6 protein (4) and the glucose transporter (5).

In the present study we have examined the regulation of protein kinase C in the perfused rat heart. α_1 -Adrenergic and muscarinic agonists known to increase phosphatidyl inositol turnover in this system (6) did not appear to translocate protein kinase C from cytosol to particulate fractions. Failure to observe agonist-induced translocation may have been due to a Ca^{2+} -induced translocation that occurred during homogenization such that particulate protein kinase C activity changed from 30% to 70% over the range of 60 nM to 4 μM free Ca^{2+} . Under homogenization conditions that maintained free Ca^{2+} at 240 nM and using extraction with 0.2% Triton X-100 (sufficient to fully extract protein kinase C from control hearts), treatment of the perfused heart with PMA resulted in a dose-dependent loss of cytosolic and particulate protein kinase C ($\text{K}_{0.5} = 10$ nM) that occurred rapidly ($t_{0.5} = 2$ min at 200 nM PMA) and which was not mimicked by 4 α -phorbol 12,13-didecanoate or diacylglycerol. As no increase in protein kinase M was observed, further investigation revealed that the apparent down-regulated protein kinase C could be recovered from the membrane fraction by vigorous extraction with 2% Triton X-100 and 10 mM EGTA.

It is concluded that activation-translocation of cardiac protein kinase C is Ca^{2+} dependent and agonist-mediated translocation if it occurs becomes only loosely bound to the particulate fraction. Tumour-promoting phorbol ester but not diacylglycerol leads to marked translocation of protein kinase C which becomes tightly bound. Phorbol ester mediated translocation can be confused with down-regulation unless vigorous membrane extraction procedures are used.

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CALCIUM-MEDIATED TRANSLOCATION OF PROTEIN KINASE C IN HOMOGENATES OF PERFUSED RAT HEART

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Hydrolysis of phosphatidyl inositol causes the production of inositol phosphates and diacylglycerol, which in many tissues results in an increase in intracellular Ca^{2+} and the activation of protein kinase C (PKC). This activation of PKC is observed as a translocation of the enzyme from the cytosol to the particulate fraction (1). Although many hormones cause phosphatidyl inositol hydrolysis in myocardial tissue (2) no activation or translocation of PKC have been reported. In the present study we have developed an assay for PKC in perfused rat heart preparations and have examined the translocation of the enzyme between cytosolic and particulate fractions.

Cytosolic and particulate extracts of PKC from Langendorff perfused rat hearts were prepared by homogenization and centrifugation (100,000 g/30 min). Cytosol extracts were further purified by ammonium sulphate precipitation and ion-exchange chromatography. Particulate extracts were purified by Triton X-100 extraction and ion-exchange chromatography. The partially purified PKC extracts were found to be Ca^{2+} , phospholipid and diolefin sensitive. The total activity extracted from the rat heart, expressed as the ability of the enzyme to phosphorylate the protein Histone H1S, was approximately 2.5 nmoles of P_i incorporated/min/g wet wt.

Unlike PKC from other tissues such as brain and skeletal muscle, the distribution of PKC between cytosolic and particulate fractions was dependent upon the concentration of Ca^{2+} in the homogenate. In hearts, which were perfused with Krebs Ringer Bicarbonate buffer containing 1.27 mM Ca^{2+} and homogenized in buffer in the absence of Ca^{2+} or EGTA, over 90% of the total activity was recovered from the particulate fraction. Addition of EGTA to the homogenizing buffer caused a redistribution of PKC activity from the particulate to the cytosolic fraction in a concentration dependent manner reaching a maximum effect at 10 mM EGTA resulting in only 20% of the activity remaining in the particulate fraction. Perfusion of the hearts with high Ca^{2+} (4 mM) increased the concentration of EGTA required to remove the enzyme from the particulate fraction. In contrast, in hearts which were perfused with 0.1 mM Ca^{2+} and homogenized in the absence of EGTA, only 50% of the total activity was associated with the particulate fraction. However, addition of Ca^{2+} (1 mM) to the homogenizing buffer resulted in redistribution so that 90% was again recovered from the particulate fraction.

These studies suggest that PKC from rat heart can be translocated between particulate and cytosolic fractions by small changes in Ca^{2+} concentrations in the homogenizing medium. Possible alterations in the distribution of PKC by hormonal stimulation are therefore obscured by changes in Ca^{2+} concentration upon homogenization. In addition these results may indicate that the translocation of the enzyme from the cytosolic to particulate fraction that occur with hormonal stimulation may be due to changes in intracellular Ca^{2+} concentrations.

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GLUCOSE-INDUCED LOSS OF EXERCISE-MEDIATED 3-O-METHYLGLUCOSE UPTAKE BY ISOLATED RAT SOLEUS AND EPITROCHLEARIS MUSCLES

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Skeletal muscle is the major site of glucose disposal in the body. However, muscle from insulin-deficient diabetic rats show a marked decrease in responsiveness to both insulin and contraction-induced glucose transport (1). Recent reports that hyperglycemia in the rat *in vivo* (2), in type I diabetics (3), and in the perfused rat hindlimb (4) induce a state of insulin resistance pose the question whether contraction-induced glucose transport is also impaired.

In this study, isolated soleus and epitrochlearis muscles (representing both red and white muscle types respectively) were incubated for 12 hr with normal (5mM) or a high (20mM) concentration of glucose and the uptake of 3-O-methylglucose in response to maximal insulin or electrical stimulation was determined. The soleus and epitrochlearis muscles were removed from anaesthetized rats and attached via the tendons to stainless steel U-shaped supports designed to maintain a tension of approx. 10g. Muscles were incubated in 5 ml of modified Krebs-Ringer saline-bicarbonate buffer which also contained 0.1% rat serum, glucose (5 or 20mM), mannitol (35 or 20mM) and insulin (100uU/ml) and the medium was changed every 4 hr. For 3-O-methylglucose determination, the muscles were remounted by the tendons in a special suspension apparatus which contained 4ml of modified Krebs-Ringer saline-bicarbonate buffer and 3-O-methyl-[1-³H]glucose. The apparatus was maintained at 37°C and gassed. Muscles were electrically stimulated by applying 200ms trains of impulses (each impulse of 1ms duration at a frequency of 50 Hz every 2 sec, adjusted 10-20 volts) to attain full fibre recruitment. Muscles were frozen (liquid N₂) until assayed for 3-O-methylglucose, ATP, ADP, AMP, creatine phosphate, lactate and glycogen determinations.

For freshly isolated soleus and epitrochlearis muscles, electrical stimulation, increased the rate of 3-O-methylglucose uptake, from 160 to 302 nmol/min/g and from 15.3 to 33.3 nmol/min/g, respectively. Muscles incubated for 12 hr with 5mM glucose responded to electrical stimulation to a similar extent as freshly excised muscles. However, for both muscle types incubation for 12 hr with 5 or 20 mM glucose led to an increase in the basal rate of 3-O-methylglucose uptake. This was greatest for the epitrochlearis muscles. Contraction-induced 3-O-methylglucose uptake was impaired in muscles that had been incubated for 12 hr with 20mM glucose. For freshly isolated soleus and epitrochlearis muscles, insulin (10mU/ml), increased the rate of 3-O-methylglucose uptake, from 160 to 272 nmol/min/g and from 15.3 to 32.8 nmol/min/g, respectively. Muscles which had not been stimulated electrically but which had been incubated for 12 hr with 5mM glucose responded to insulin to the same extent as freshly excised muscle. Soleus muscles incubated for 12 hr with 20mM glucose resulted in a loss of insulin responsiveness (from 190 to 210 nmol/min/g). Epitrochlearis muscles were unaffected. These findings extend the observations of Richter *et al.* (4) by showing that prolonged exposure of skeletal muscle to high levels of glucose not only induces a state of insulin resistance but also a resistance to exercise-induced 3-O-methylglucose uptake.

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EFFECTS OF PMA ON PROTEIN KINASE C IN PERFUSED RAT HEART

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Protein kinase C (PKC), the phospholipid, Ca²⁺-dependent protein kinase has been implicated in cardiac hypertrophy (Simpson *et al.* *J. Molec. Cell. Cardiol.* 18, suppl. 5, 45-58, 1985) and more recently in cardiac glucose transport (Van de Werve *et al.* *Diabetes* 36, 310-314, 1987). In addition α_1 adrenergic agonists as well as muscarinic agonists increase phosphatidylinositol (PI) turnover in heart thus implying a role for PKC as part of the signal transduction processes. α -Adrenergic agonists also activate cardiac trans-sarcolemma electron efflux (Low *et al.* *Biochim. Biophys. Acta* 844, 142-148, 1985). Since neither PI-turnover nor electron efflux is affected by β -agonists it appeared possible that PKC activation and electron efflux were uniquely involved in α -adrenergic receptor activation and were causally related. In the present study three questions were addressed: - (i) α_1 -adrenergic receptor activation associated with PKC activation?; (ii) what is the effect of treatment of the perfused rat heart with tumour-promoting phorbol ester (PMA), a putative activator of PKC?; and (iii) what is the relationship between PKC and trans-sarcolemma electron efflux?

The system used was the Langendorff perfused rat heart. Perfusions were conducted at 37°C and employed Krebs Henseleit bicarbonate buffer containing 5 mM glucose, 1.27 mM Ca²⁺ and 0.05 mM EDTA. Hearts were homogenized in Ca²⁺/EGTA buffers to set the free [Ca²⁺] at 230 nM. Protein kinase activity was determined following anion exchange chromatography on DE-52 of cytosol and either 0.2% or 2% Triton X-100 extracts of the 100,000 g particulate material using histone III_s as substrate.

Initial experiments failed to show α_1 adrenergic-mediated activation of PKC as judged by the failure of 1 μ M epinephrine + 10 μ M DL propranolol to cause translocation from cytosol to membrane fractions. Activation of PKC was

masked by a Ca²⁺-induced translocation that occurred during homogenization such that particulate PKC changed from 33.5% to 78% over the range of 20 nM to 2 μ M free Ca²⁺. Treatment of the perfused heart with PMA and extraction with 0.2% Triton X-100, sufficient to fully extract PKC activity from control hearts, resulted in a dose-dependent apparent loss of total PKC (K_{0.5} = 6 nM) that occurred rapidly (t_{0.5} = 1.5 min at 200 nM) and was not mimicked by non-tumour promoting phorbol ester. However extraction of the particulate material with 2% Triton X-100 showed that PMA caused a tighter binding of PKC and not a loss of activity. PMA did not stimulate trans-sarcolemma electron efflux but the 60% apparent loss of total PKC activity coincided with the total loss of α_1 adrenergic stimulation of electron efflux and inotropy. PKC would appear to be involved in α_1 adrenergic control of contractility but not causally related to sarcolemmal electron efflux.

(Nato Advanced Research Workshop:
Spain, 1988. p. P21)